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Endothelial cell modulation of smooth muscle contraction by gap junctional communication and vascular autacoids

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Endothelial Cell Modulation of Smooth Muscle Contraction by Gap Junctional Communication and Vascular Autacoids

Submitted by Lorraine Anne Frances McEvoy

for the degree of PhD

of the University of Bath

2004



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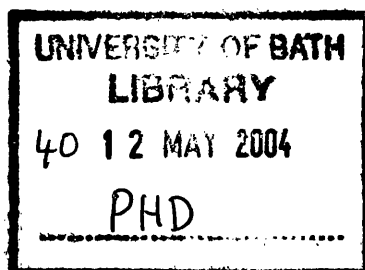
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Abstract

1 Communication between cells of the artery wall co-ordinate and modulate changes in vessel diameter. Experiments were designed to investigate the role of intercellular gap junctions versus extracellular signalling mechanisms in the communication of changes in $[Ca^{2+}]_i$ and membrane potential between isolated endothelial cells and between the endothelium and smooth muscle of intact arteries.

2 In cultured monolayers of rat aortic endothelial cells, application of a discrete mechanical stimulus to a single cell, evoked an increase in $[Ca^{2+}]_i$ within the stimulated cell which spread to neighbouring non-stimulated cells.

3 Neither the gap junction uncoupling agent palmitoleic acid (50 μ M), the Ca^{2+} -ATPase inhibitor cyclopiazonic acid (10 μ M) nor the phospholipase C inhibitor U73122 (10 μ M), affected the mechanically-evoked increase in $[Ca^{2+}]_i$ within the stimulated cell, yet in each case, increases in $[Ca^{2+}]_i$ within the non-stimulated cells were abolished. Removal of extracellular Ca^{2+} inhibited mechanically-evoked increases in $[Ca^{2+}]_i$ within the stimulated cell, while Ca^{2+} responses by the non-stimulated cells were unaffected. These data indicate that the intercellular passage, *via* gap junctions, of a Ca^{2+} -mobilizing messenger other than Ca^{2+} itself, underlies the communication of changes in $[Ca^{2+}]_i$ between vascular endothelial cells.

4 In rat mesenteric arteries mounted in a wire myograph and contracted with phenylephrine (0.1 – 10 μ M), acetylcholine (3 nM – 10 μ M) evoked concentration-dependent EDHF-mediated relaxation. Relaxation was not influenced by the level of contraction (low <70% of tissue maximum, high 70% - 100% of tissue maximum).

5 The gap junction uncoupling agents carbenoxolone (100 μ M) and Gap 27 (300 μ M), were without effect on EDHF-mediated relaxation to acetylcholine against low levels of phenylephrine-evoked contraction, but completely abolished and attenuated respectively, relaxation to acetylcholine at high levels of contraction. These data provide evidence that as arterial contraction increases, the dependence on gap junctional signalling for EDHF-mediated relaxation increases.

6 In rat mesenteric arteries mounted in a wire myograph, inhibition of nitric oxide synthase and soluble guanylate cyclase with L-NAME (100 μ M) and ODQ (10 μ M) respectively, significantly augmented phenylephrine-evoked contraction in an endothelium-dependent manner. L-NAME-evoked augmentation of phenylephrine contraction was blocked by the general ABC protein inhibitor, glibenclamide (10 μ M), while carbenoxlone (100 μ M) and Gap 27 (300 μ M) were without effect.

7 Intact rat mesenteric arteries released ATP into the extracellular milieu in response to stimulation with phenylephrine (10 μ M). Glibenclamide (10 μ M) abolished phenylephrine-evoked ATP release.

8 In isolated mesenteric smooth muscle and endothelial cells loaded with the Ca^{2+} indicator fluo-4 AM (50 μ M), phenylephrine (10 μ M) stimulation evoked an increase in fluorescence in smooth muscle but not endothelial cells. ATP (10 μ M) evoked an increase in fluorescence in both smooth muscle and endothelial cells.

9 These data suggest that α_1 -adrenoceptor stimulation of rat mesenteric arteries is associated with the release of ATP from smooth muscle by a mechanism involving ABC proteins. Endothelial cell activation by ATP released from smooth muscle, independent of gap junctions, appears to underlie phenylephrine-evoked nitric oxide release in this artery.

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List of abbreviations

ABC proteins	ATP binding cassette proteins
ACh	Acetylcholine
Ac-LDL	Acetylated-low density lipoprotein
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
BK _{ca}	Large conductance calcium-activated potassium channel
BSA	Bovine serum albumin
[Ca ²⁺] _i	Intracellular calcium concentration
cAMP	Cyclic adenosine 3',5'-cyclic monophosphate
CBX	Carbenoxolone
cGMP	Cyclic guanosine 3',5'-cyclic monophosphate
CGRP	Calcitonin gene-related peptide
ChTx	Charybdotoxin
COX	Cyclooxygenase
CPA	Cyclopiazonic acid
DAG	Diacylglycerol
DEA NONOate	Diethylamine NONOate
DiI	1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate
DMEM	Dulbeccos modified Eagle medium
DMSO	Dimethyl sulphoxide
DTT	Dithiothrietol
ECAF	Endothelial cell attachment factor
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
EETs	Epoxyeicosatrienoic acids
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N',tetraacetic acid
EtOH	Ethanol
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
GA	Glycyrrhetic acid
GJC	Gap junction channel
GTP	Guanosine 5'-triphosphate
IEL	Internal elastic lamina
IbTx	Iberiotoxin
IK _{ca}	Intermediate conductance calcium-activated potassium channel
IP ₃	Inositol 1,4,5-trisphosphate
IP ₃ R	Inositol 1,4,5-trisphosphate receptors
[K ⁺] _o	Extracellular potassium concentration
K _{ATP}	ATP-modulated potassium channel
K _{ca}	Calcium-activated potassium channel
K _{IR}	Inward-rectifying potassium channel
L-NAME	N ^ω -nitro-L-arginine methyl ester
L-NOARG	L-N ^G nitoarginine

α,β -MeATP	α,β -methylene-ATP
$[Mg^{2+}]_i$	Intracellular calcium concentration
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NA	Noradrenaline
$[Na^+]_i$	Intracellular sodium concentration
NEM	N-ethylmaleimide
NGS	Normal goat serum
NO	Nitric oxide
NOS	Nitric oxide synthase
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
PA	Palmitoleic acid
PBS	Phosphate buffered saline
PE	Phenylephrine
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PGI ₂	Prostacyclin
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PLA	Propyl-L-arginine
PLC	Phospholipase C
R_{max}	Maximum relaxation
SAC	Stretch-activated channels
S.E.M	Standard error of mean
SERCA	Sarcoplasmic reticulum Ca^{2+} -ATPase
sGC	Soluble guanylate cyclase
SK _{ca}	Small conductance calcium-activated potassium channel
SR	Sarcoplasmic reticulum
vWF	Von Willebrand factor

Published work arising from this thesis

DORA K.A, McEVOY L.A.F, KING M, INGS N & GARLAND C.J (2003). Agonist-Stimulation Intensity Influences the Mechanism for Relaxation in Rat Mesenteric Arteries. In *EDHF 2002*. ed. Vanhoutte P.M. pp. 256-260. London: Taylor and Francis.

McEVOY L.A.F & DORA K.A (2002). Intercellular Calcium Signalling via Gap Junctions in Response to Mechanosensitive Signals in Rat Aortic Endothelial Cells. *Br. J. Pharmacol.*, **135**. supp. 119P.

McEVOY L.A.F, GARLAND C.J & DORA K.A (2002). Phospholipase C Dependent Intercellular Calcium Signalling via Gap Junction Channels in response to Mechanosensitive Signals in Rat Aortic Endothelial cells. *The Pharmacologist*. **44** (2) supp.1.130.22.

CHAPTER ONE

Introduction

1.1 Structure and function of small resistance arteries

Resistance arteries are vessels of the cardiovascular system that contribute significantly to precapillary resistance. Arteriolar resistance is determined predominantly by arterioles and by those vessels proximal to the arterioles, small arteries, often generally defined as vessels with a diameter of up to 400 μm (Christensen & Mulvany, 2001; Mulvany & Aalkjaer, 1990). The mechanisms by which small arteries regulate changes in lumen diameter and thus vascular resistance, are therefore relevant to our understanding of the control of blood flow and tissue perfusion. Arteries of the mesenteric arcade are considered true resistance vessels fundamental to the control of intestinal blood flow (Christensen & Mulvany, 1993; Fenger-Gron *et al.*, 1997) and are consequently an appropriate vascular preparation in which to study vasomotor responses in resistance arteries.

1.1.1 Arterial morphology

All arteries follow a common plan of histological organisation consisting of three concentric layers or 'tunics', an outer tunica adventitia, a central tunica media and an inner tunica intima. The adventitial layer is comprised principally of longitudinally orientated collagen and elastic fibres. Nerve fibres of the autonomic nervous system are associated with this layer and consist of sympathetic vasoconstrictor neurones, parasympathetic vasodilator neurones and sensory motor mechanisms. Tunica media is bounded on the luminal side by an internal elastic lamina (IEL) and consists of concentric layers of helically arranged smooth muscle cells. Smooth muscle cells are

the prevailing component of muscular arteries and arterioles and ultimately regulate changes in blood flow through contraction or relaxation and the consequent changes in vessel diameter. The perivascular nerves of the adventitia do not generally penetrate to this layer, so released neurotransmitter must diffuse for several microns to affect smooth muscle cells of the media. The innermost layer lining the internal surface of the vessel, tunica intima, consists of a layer of endothelial cells resting on the IEL. Endothelial cells of small arteries frequently project through fenestrations in the IEL to make contact with smooth muscle cells within the tunica media (for reviews see (Mulvany & Aalkjaer, 1990; Pugsley & Tabrizchi, 2000). The close apposition between smooth muscle and endothelial cells as a result of endothelial projections through the IEL, allows the potential for intercellular communication between the two cell types (Sandow & Hill, 2000; Sandow *et al.*, 2002). A morphological basis for intercellular communication is provided within the wall of most arteries by the presence of gap junction channels between cells.

Gap junctions are intercellular channels concentrated in specialised contact regions of the plasma membrane and form a direct link between cytosolic compartments of physically adjacent cells (Brink, 1998; Evans, 1997; Yeager, 1998). A complete gap junction channel is formed by the association of two connexon hemichannels, each one contributed by different but neighbouring cells. A connexon hemichannel is constructed from six connexin protein subunits which assemble to form a central aqueous pore and permit the exchange of ions and small molecules of up to 1 kDa in mass (Evans, 1997).

At least 14 connexin proteins with molecular weights ranging from 26 to 70 kDa have been cloned (Beyer *et al.*, 1990). The nomenclature generally adopted to distinguish

between the individual subtypes is the molecular weight of the protein (Beyer *et al.*, 1990). Each connexin is composed of four α -helical membrane-spanning domains, two extracellular loops, an intracellular loop and intracellular N- and C- termini. The extracellular loops are highly conserved between connexins and likely to be instrumental in the docking of two connexon hemichannels (Evans, 1997; Yeager, 1998). The different molecular masses of the connexins are due to changes in the length of the C-terminus (Dhein, 1998), the part of the protein also concerned with modulating gap junction conductance by virtue of the numerous phosphorylation sites within this domain (Yeager, 1998). Connexin phosphorylation by protein kinase C (PKC), cAMP-dependent protein kinase A (PKA), protein kinase G (PKG), tyrosine kinases and mitogen-activated (MAP) kinase may increase or decrease gap junctional conductance, the latter effect also being associated with reductions in intracellular pH and increases in $[Ca^{2+}]_i$, $[Mg^{2+}]_i$ and $[Na^+]_i$ (Dhein, 1998).

Gap junction channels are considered homotypic, when the connexon hemichannels are composed entirely from the same connexin subtype, heterotypic, when each connexon is composed from a single but differing connexin subtype and heteromeric when each connexon is composed from more than one connexin subtype (Brink, 1998). In vitro, functional homotypic and heterotypic channels can be formed between all combinations of vascular connexins (Elfgang *et al.*, 1995; Valiunas *et al.*, 2000). However, the ability of tracer molecules of differing size and charge to permeate gap junctions varies according to the connexin composition of the channel (Elfgang *et al.*, 1995). Such diversity in gap junction composition may confer unique properties upon individual channels (Delmar, 2002).

Connexin proteins 37, 40, 43 and 45 are expressed in vascular smooth muscle (Christ *et al.*, 1996; Li & Simard, 2001; Nakamura *et al.*, 1999), while endothelial cells have been found to express connexins 37, 40 and 43 only (Carter *et al.*, 1996; Christ *et al.*, 1996). However, heterogeneity exists between vessels and species with regard to the distribution of gap junctions and their constituent connexins (Hill *et al.*, 2002; van Kempen & Jongsma, 1999; Yeh *et al.*, 1998). Connexin expression within both cell types potentially permits the formation of functional gap junction channels between smooth muscle cells, between endothelial cells and between smooth muscle and endothelial cells.

In rat mesenteric arteries, serial-section electron microscopy has revealed the presence of gap junctions between cells of the endothelial layer and between smooth muscle and endothelial cells (Sandow & Hill, 2000; Sandow *et al.*, 2002). Immunofluorescent labelling of these arteries with antibodies for the three major vascular connexins (37, 40 and 43) has revealed the expression of all three proteins within these arteries (Gustafsson *et al.*, 2003). Connexin 43 has generally been considered to be the predominant connexin expressed by vascular smooth muscle (Severs *et al.*, 2001), although its expression within the media of the rat vasculature is largely restricted to elastic arteries, such as the aorta (Hong & Hill, 1998). Indeed, immunocytochemical studies have located connexins 37, 40 and 43 within the endothelium of the rat aorta, while connexin 43 was the only connexin protein expressed by aortic smooth muscle cells (Hill *et al.*, 2002).

1.2 Regulation of vascular tone

Changes in arterial diameter of small arteries and arterioles determine peripheral resistance and the extent of tissue perfusion. Interactions between all three of the main regulatory cell types of the vascular wall: endothelial cells, smooth muscle cells and perivascular nerves, contribute to the local modulation of vascular tone, with the smooth muscle ultimately regulating vessel diameter.

1.2.1 Arterial constriction

Constriction of vascular smooth muscle depends principally upon two variables; the concentration of intracellular calcium ($[Ca^{2+}]_i$) and the sensitivity of the contractile proteins to calcium (Karakci *et al.*, 1997). Ca^{2+} entry across the plasma membrane and/or Ca^{2+} release from intracellular stores provide the necessary increase in $[Ca^{2+}]_i$ to initiate a contractile response. Resting membrane potential is the principal determinant of calcium influx across the plasma membrane, with membrane depolarization activating voltage gated L-type Ca^{2+} channels to allow extracellular calcium influx (Nelson *et al.*, 1988). Agonist-evoked stimulation of $G_{q/11}$ -protein linked receptors coupled to the enzyme phospholipase C (PLC), releases Ca^{2+} from intracellular stores through PLC mediated phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis and consequent production of the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Binding of IP_3 to its receptors present on the sarcoplasmic reticulum initiates Ca^{2+} release from these stores (Berridge, 1993). Concomitant membrane depolarization *via* Cl^- efflux in response to activation of $G_{q/11}$ -protein linked

receptors, contributes further to agonist-evoked increases in smooth muscle cell calcium (Nilsson, 1998).

The link between an increase in smooth muscle cell calcium and contraction is provided by the enzyme myosin light chain kinase (MLCK), which phosphorylates the 20 kDa light chain (MLC) of the contractile protein myosin (Karaki *et al.*, 1997). Ca^{2+} regulates MLCK activity in a calmodulin-dependent manner, a rise in $[\text{Ca}^{2+}]_i$ increasing MLCK activity causing phosphorylation of MLC thereby enabling its interaction with actin and initiation of the contractile process (Karaki *et al.*, 1997).

Calcium sensitisation, a phenomenon described as the enhancement of contraction at a given $[\text{Ca}^{2+}]_i$, also evokes constriction of vascular smooth muscle and can be initiated by stimulation of $\text{G}_{q/11}$ -protein linked receptors (Wier & Morgan, 2003). Increased MLC phosphorylation as a result of downregulated myosin phosphatase activity and/or Ca^{2+} -calmodulin-independent phosphorylation, have been elucidated as the molecular basis for calcium sensitization (Karaki *et al.*, 1997). DAG evoked PKC activation (Buus *et al.*, 1998) and G-protein mediated activation of the small GTPase Rho (Ito *et al.*, 2003) are believed to mediate the calcium sensitising response.

In vivo, co-release of noradrenaline (NA) and adenosine 5'-triphosphate (ATP) from sympathetic nerve terminals of the rat small mesenteric artery, initiate vasoconstriction predominantly mediated by activation of smooth muscle $\text{G}_{q/11}$ -protein linked α_{1A} -adrenoceptors (Williams & Clarke, 1995) and ligand gated P_{2X1} purinoceptors respectively (Hansen *et al.*, 1999). The relative contribution of each transmitter to the contractile response varies greatly between vascular beds (Ralevic & Burnstock, 1998).

In the rat mesenteric bed, release of NA is the predominant mechanism by which neurogenic contractions of these arteries are evoked (Lamont *et al.*, 2003).

In all functional studies presented in this thesis, vascular preparations were contracted with the α_1 -adrenoceptor agonist phenylephrine (PE). The use of this agonist enabled increases in arterial tone to be evoked in a way that closely resembles vasoconstriction *in vivo*.

1.2.2 Arterial relaxation

In converse to vasoconstriction, relaxation of vascular smooth muscle is evoked by decreases in intracellular calcium or by decreases in the sensitivity of the contractile apparatus for calcium. Reduced calcium entry or release, in addition to increased calcium extrusion or uptake, will lower smooth muscle intracellular Ca^{2+} in response to the release of vasoactive factors from the endothelium, or direct receptor-mediated activation of the smooth muscle.

1.2.2.1 Endothelium-dependent vasodilatation

Since the discovery that endothelial cells can modulate smooth muscle tone through the production and release of a diffusible endothelium-derived relaxing factor (EDRF, Furchgott & Zawadski, 1980) later identified as nitric oxide (NO, Ignarro *et al.*, 1987; Palmer *et al.*, 1987) much interest has been focused on how interactions between these two cell types control blood vessel diameter.

Nitric oxide

NO is generated from precursor L-arginine (Palmer *et al.*, 1988) by a family of enzymes known as the nitric oxide synthases (NOS). Three isoforms of NOS have been identified and characterised based on cellular distribution and regulation: neuronal NOS (I), inducible NOS (II) and endothelial NOS (III) also abbreviated as nNOS, iNOS and eNOS respectively (Alderton *et al.*, 2001). The constitutively expressed, membrane-bound eNOS isoform, present in vascular endothelial cells, is largely responsible for the production of endothelium-derived NO (Govers & Rabelink, 2001). Calmodulin was the first protein shown to interact with NOS (Bredt & Snyder, 1990), the dependency of the interaction distinguishing between NOS isoforms, with nNOS and eNOS having a much higher requirement for Ca^{2+} than iNOS (Alderton *et al.*, 2001). The ability of a rise in $[\text{Ca}^{2+}]_i$ to activate eNOS is such that endothelial receptors coupled to calcium mobilization will evoke the production and release of NO (Fleming & Busse, 1999; Govers & Rabelink, 2001), a mechanism commonly exploited experimentally to release endogenous NO from vascular endothelium. However, recent studies have demonstrated that eNOS can be activated in a Ca^{2+} -independent manner by phosphorylation with serine/threonine kinase Akt (protein kinase B (PKB); Dimmeler *et al.*, 1999; Fulton *et al.*, 1999). Indeed, fluid shear stress, the primary physiological stimulus for the release of NO from vascular endothelium, can activate eNOS by a Ca^{2+} -independent mechanism (Fisslthaler *et al.*, 2000; Fleming *et al.*, 1998). These findings may explain why maintained exposure of endothelial cells to fluid shear stress results in the sustained production of NO (Ayajiki *et al.*, 1996), while the shear stress evoked rise in $[\text{Ca}^{2+}]_i$ is transient (Mo *et al.*, 1991; Shen *et al.*, 1992). The cellular and molecular pathways mediating shear stress-evoked NO release are unclear, but may involve

activation of platelet-endothelial cell adhesion molecule-1 (PECAM-1) as eNOS and PECAM-1 have been shown to be co-localized within endothelial cells (Andries *et al.*, 1998) and phosphorylation of PECAM-1 can occur in response to physical stimuli (Osawa *et al.*, 1997). Other cell components that may play a role in shear stress-induced eNOS activation include caveolae, through the localisation of signalling molecules involved in mechanotransduction (Rizzo *et al.*, 1998) and the cytoskeleton (Hutcheson & Griffith, 1996). In a similar manner, eNOS activation by isometric vessel contraction has also been demonstrated to be independent of calcium (Fleming *et al.*, 1999).

The ability of NOS inhibitors to augment vasoconstrictor-evoked responses in a range of vascular preparations, demonstrates a basal or vasoconstrictor-stimulated release of nitric oxide (Boer *et al.*, 1999; Dora *et al.*, 1997; Dora *et al.*, 2000; Tuttle & Falcone, 2001). In rat mesenteric arteries, the finding that PE-evoked contraction was augmented by the NOS inhibitor L-NAME and that L-NAME was without effect on tension under basal conditions, suggested that smooth muscle activation with PE evokes release of endothelial NO (Dora *et al.*, 2000). Due to the absence of tone in these arteries prior to the application of phenylephrine, the possibility that a basal release of NO may underlie these observations must be considered. Indeed, measurements of cyclic guanosine-3',5'-monophosphate (cGMP) formation suggest a low basal level of NO release in rat mesenteric arteries (Plane *et al.*, 1996). However, the 2 - 3 fold increase in cGMP formation seen following the addition of phenylephrine, suggests that NO release in rat mesenteric arteries is evoked in response to a vasoconstrictor stimulus (Plane *et al.*, 1996). The mechanism underlying vasoconstriction-evoked NO release in rat mesenteric arteries has yet to be elucidated, although PE has been shown to indirectly stimulate increases in endothelial $[Ca^{2+}]_i$ within these arteries (Schuster *et al.*, 2001).

Upon diffusion to the smooth muscle, the chief mechanism by which NO evokes vasodilatation is by activation of the haeme-containing enzyme soluble guanylate cyclase (sGC), which catalyses the conversion of guanosine-5'-triphosphate (GTP) to cGMP. The subsequent activation of smooth muscle cGMP-dependent protein kinase (PKG) is ultimately responsible for the majority of cGMP-mediated relaxation responses in vascular smooth muscle (Lincoln *et al.*, 1994). A number of mechanisms contribute to the relaxant effects of PKG. Inhibition of L-type calcium channels and increased plasmalemmal Ca^{2+} -ATPase activity by PKG-mediated phosphorylation events relax smooth muscle by lowering the $[\text{Ca}^{2+}]_i$ (Ishikawa *et al.*, 1993; Rashatwar *et al.*, 1987; Xiong *et al.*, 1994). Increased Ca^{2+} sequestration by the sarcoplasmic reticulum (SR) in response to PKG-mediated increases in sarcolemmal Ca^{2+} -ATPase activity also contributes to the $[\text{Ca}^{2+}]_i$ lowering effect of the kinase (Cornwell *et al.*, 1991). PKG evoked Ca^{2+} -desensitization and activation of smooth muscle K^+ channels (Nishimura & Van Breemen, 1989; Robertson *et al.*, 1993) provide additional parallel mechanisms for PKG-mediated relaxation of vascular smooth muscle. In rat mesenteric arteries, NO may also evoke relaxation of vascular smooth muscle independently of PKG, by directly activating smooth muscle large-conductance Ca^{2+} -activated K^+ channels (BK_{ca} , Mistry & Garland, 1998; Plane *et al.*, 1996) and delayed rectifier potassium channels (Plane *et al.*, 2001).

Amino acid derivatives are commonly used agents to pharmacologically inhibit NOS enzyme activity and block NO synthesis. Guanidino substituted analogues of L-arginine such as L-*N*^Gnitroarginine (L-NOARG) and its methyl ester L-NAME, are potent inhibitors of NOS both *in vitro* and *in vivo* by competing with L-arginine for substrate

binding sites (Alderton *et al.*, 2001; Moore & Handy, 1997). Inhibition of sGC with the potent and selective agent 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), blocks the PKG-mediated actions of NO without affecting NO synthesis (Hobbs, 1997).

Prostacyclin

Prostacyclin (PGI₂) is synthesised within endothelial cells by cyclooxygenase (COX)-mediated arachidonate metabolism. PGI₂ liberated by the endothelium, activates IP-prostanoid receptors present on smooth muscle cells (Narumiya *et al.*, 1999) and by stimulating the synthesis of adenosine 3',5'-cyclic monophosphate (cAMP), induces vasodilatation (Kukovetz *et al.*, 1979). In a similar manner to that of cGMP, cAMP induced extrusion of cytosolic Ca²⁺ and decreases in the sensitivity of the contractile apparatus for Ca²⁺, mediate the relaxation response (Abe & Karaki, 1992). However, PGI₂ does not play a significant role as an endothelium-derived dilator in rat mesenteric arteries (Garland & McPherson, 1992).

Endothelium-derived hyperpolarizing factor

The existence of a third endothelium-derived dilator was proposed following the discovery of responses insensitive to inhibitors of NOS and COX. Dilator responses to this factor, now defined by their insensitivity to inhibitors of NOS and COX, have been demonstrated to be associated with vascular smooth muscle hyperpolarization, resulting in the term endothelium-derived hyperpolarizing factor (EDHF, Chen *et al.*, 1988; Feletou & Vanhoutte, 1999; Taylor & Weston, 1988).

In general, hyperpolarization of smooth muscle produces relaxation by reducing the open probability of voltage-dependent Ca^{2+} channels, thereby decreasing calcium influx and lowering intracellular calcium levels (Nelson *et al.*, 1990). The significant role of smooth muscle depolarization resulting from Ca^{2+} -dependent chloride current activation (Nelson *et al.*, 1990), and the associated entry of extracellular Ca^{2+} via L-type Ca^{2+} channels in agonist-induced contraction of smaller arteries (Nilsson, 1998), may explain why the relative importance of EDHF compared with other EDRFs increases with decreasing vessel size (Hwa *et al.*, 1994; Shimokawa *et al.*, 1996; Tomioka *et al.*, 1999).

While the identity of the hyperpolarizing factor that mediates smooth muscle relaxation remains controversial, increases in endothelial cell calcium by both receptor-dependent and -independent mechanisms play a pivotal role in the initiation of EDHF-mediated responses (Chen & Suzuki, 1990; Fukao *et al.*, 1995). Putative candidates include the cytochrome P_{450} -derived epoxyeicosatrienoic acids (EETs, Campbell *et al.*, 1996; Campbell & Harder, 1999), endocannabinoids (Randall *et al.*, 1996), potassium released from endothelial cells (Edwards *et al.*, 1998) and the transmission of hyperpolarization from the endothelium to the smooth muscle *via* gap junctions (Dora & Garland, 1999; Sadow & Hill, 2000; Yamamoto *et al.*, 1998). However, the identity of a universal hyperpolarizing factor is unlikely, with current evidence favouring the existence of multiple EDHF's with a degree of species and tissue specialization (for reviews see: Busse *et al.*, 2002; Feletou & Vanhoutte, 1999; Garland *et al.*, 1995; Mombouli & Vanhoutte, 1997).

In rat mesenteric arteries, a hallmark of the EDHF-mediated response is its abolition in the presence of the small-conductance Ca^{2+} -activated K^+ channel (SK_{ca}) inhibitor apamin, combined with charybdotoxin, an inhibitor of both BK_{ca} and intermediate-conductance Ca^{2+} -activated K^+ channels (IK_{ca} , Doughty *et al.*, 1999; Waldron & Garland, 1994). The more recent finding that together, apamin and the selective inhibitors of IK_{ca} , TRAM-39 (Hinton & Langton, 2003) and TRAM-34 (Crane *et al.*, 2003), inhibit EDHF-mediated relaxation and hyperpolarization, suggests that activation of SK_{ca} and IK_{ca} are pivotal to the EDHF-response in this artery. The expression profile for IK_{ca} and SK_{ca} in the rat mesenteric artery indicates that these channels are confined to the endothelium (Dora & Garland, 1999; Doughty *et al.*, 1999; Hinton & Langton, 2003; Walker *et al.*, 2001), whereas BK_{ca} are found only in the smooth muscle cell layers (Walker *et al.*, 2001). Thus, as endothelial cell hyperpolarization is a crucial early step in the EDHF pathway (Edwards *et al.*, 1998; Hinton & Langton, 2003; White & Hiley, 2000), the efflux of K^+ which underlies this hyperpolarization, has been suggested to make an important contribution to the EDHF-evoked vascular relaxation (Burnham *et al.*, 2002; Dora *et al.*, 2002; Edwards *et al.*, 1998). Indeed, in rat mesenteric arteries, raising the extracellular K^+ concentration by up to 12 mM evokes smooth muscle hyperpolarization and relaxation by activating an ouabain-sensitive Na^+/K^+ -ATPase and a Ba^{2+} -sensitive inwardly rectifying K^+ current (K_{IR}), mimicking an EDHF response (Dora & Garland, 2001; Edwards *et al.*, 1998). However, the proposal by Edwards *et al.* (1998) that in rat mesenteric arteries, EDHF-type responses are in part attributable to the diffusion of endothelium-derived K^+ to the smooth muscle, has been refuted by other studies in which the characteristics of K^+ -induced hyperpolarization and relaxation differed from that of EDHF (Doughty *et al.*, 2000; Lacy *et al.*, 2000). Where K^+ has failed to mimic EDHF-responses, a role for myoendothelial gap junctions

in the spread of hyperpolarization from the endothelium to the smooth muscle has been suggested (Doughty *et al.*, 2000), an idea strengthened by morphological evidence of the presence of myoendothelial gap junctions in rat mesenteric arteries (Sandow & Hill, 2000; Sandow *et al.*, 2002). The transfer of hyperpolarizing current between the two cell types has been shown to be sensitive to gap junction uncoupling agents such as glycyrrhetic acid derivatives or connexin mimetic peptides in many arteries (Dora *et al.*, 1999; Edwards *et al.*, 1999; Sandow *et al.*, 2002; Yamamoto *et al.*, 1999). An increase in the number of myoendothelial gap junctions with decreasing arterial size within the mesenteric bed (Sandow & Hill, 2000; Sandow *et al.*, 2002), in parallel to the changing importance of EDHF in the control of vascular tone (Shimokawa *et al.*, 1996), is in further support of a role for these channels in the EDHF-response.

1.2.2.2 Endothelium-independent vasodilatation

Capsaicin-sensitive sensory motor nerves contribute to the regulation of vascular tone in rat mesenteric arteries by the release of the potent vasodilator peptide calcitonin gene-related peptide (CGRP, Kawasaki *et al.*, 1988) and neuronal NO (Ferrer *et al.*, 2000; Marin & Balfagon, 1998). Arterial relaxation to CGRP is typically due to activation of smooth muscle CGRP₁ receptors and a decrease in intracellular Ca²⁺ regulated by adenylate cyclase (Bell & McDermott, 1996). Neurogenic vasodilatation can also be evoked by sympathetic nerve stimulation in response to activation of adenylate cyclase-coupled β -adrenoceptors located on vascular smooth muscle (Guimaraes & Moura, 2001; Johnson, 1998; Kawai & Koss, 1999; Phillips *et al.*, 2000). However, the endothelium, *via* the release of NO, is now believed to play a role in facilitating

relaxation to β -adrenoceptor agonists in a number of arteries (Brawley *et al.*, 2000; Toyoshima *et al.*, 1998; Xu *et al.*, 2000). In the rat mesentery, relaxation in response to β -adrenoceptor activation is only partly attenuated by NOS inhibition (Graves & Poston, 1993), the remainder of which is likely to be endothelium-independent.

1.3 Intercellular communication between vascular cells

Intercellular communication between arterial cells plays an important role in the control of vessel diameter. Transmission of intercellular signals *via* gap junctions and the release of diffusible signalling molecules, provide the pathways by which these cells interact to modulate vascular tone.

1.3.1 Gap junctions

Intercellular signals that may be transferred between cells *via* gap junctions include a variety of second messengers; cAMP (Bedner *et al.*, 2003), Ca^{2+} (Christ *et al.*, 1992) and IP_3 , (Boitano *et al.*, 1992; Carter *et al.*, 1996; Saez *et al.*, 1989; Sneyd *et al.*, 1998) and the electrotonic spread of current (Emerson & Segal, 2000a; Emerson & Segal, 2000b). Such direct intercellular transfer of signalling molecules, ions and electrical signals, is a potential mechanism whereby changes in second messenger concentration or membrane potential may be communicated between cells, including those of the vascular wall.

To establish the role of gap junctions in the communication of intercellular signals, the use of gap junction uncoupling agents such as heptanol, glycyrrhetic acid (GA) and its water-soluble derivative carbenoxolone, palmitoleic acid (PA) and connexin mimetic peptides are frequently employed (Rozental *et al.*, 2001). Lipophilic agents such as heptanol (Bastiaanse *et al.*, 1993; Christ *et al.*, 1991; Kimura *et al.*, 1995), palmitoleic acid (Burt *et al.*, 1991) and GA, a steroidal aglycone derived from glycyrrhizic acid, a

compound found in the liquorice root *glycyrrhizia glabra* (Goldberg *et al.*, 1996; Guan *et al.*, 1996; Guo *et al.*, 1999), are thought to be incorporated into the membrane and uncouple gap junctions in a physical manner by acting at the protein-lipid boundaries of the gap junction channels (Brink, 1998). More specifically, heptanol may uncouple gap junctions by decreasing the fluidity of the cholesterol rich domains of the membrane, within which the gap junction channels are embedded (Bastiaanse *et al.*, 1993). GA exists in α and β isoforms, both of which have been shown to be gap junction inhibitors (Davidson *et al.*, 1986). However, the β isoform may also depress vascular smooth muscle tone (Taylor *et al.*, 1998; Yamamoto *et al.*, 1999), while the water-soluble derivative of this isoform, carbenoxolone appears to have fewer non-specific effects. Non-specific effects of heptanol as a gap junction inhibitor, such as the reported depression of vascular smooth muscle tone (Chaytor *et al.*, 1997), may result from altered properties of the cell membrane (Takens-Kwak *et al.*, 1992). The connexin-mimetic peptides are synthetic peptides with specific sequence homology to regions of one of the extracellular loops of a particular connexin protein or proteins. ^{37,43}Gap 27 is a connexin mimetic peptide (sequence SRPTEKTIFII) homologous to the second extracellular loop region (termed Gap 27) of connexins 37 and 43 (indicated by superscript numbering) and can therefore be used to uncouple gap junction channels composed from these connexins. The mechanism of action of the connexin-mimetic peptides is unknown, but they may prevent connexon docking or interfere with intact gap junctions (Rozental *et al.*, 2001). ^{37,43}Gap 27 will be referred to as Gap 27 for the rest of this thesis.

In a number of vascular beds, lucifer yellow dye transfer and electrophysiological measurements of conducted hyperpolarization between smooth muscle and endothelial

layers indicate that functional gap junction channels connect these two cell types (Beny & Connat, 1992; Emerson & Segal, 2000a; Emerson & Segal, 2000b; Yamamoto *et al.*, 1999). An important role for these channels in EDHF-mediated vasodilatation has been demonstrated in rat mesenteric arteries, where there is a correlation between the incidence of myoendothelial gap junctions and the role of EDHF relative to NO (Sandow & Hill, 2000; Shimokawa *et al.*, 1996). The ability of Gap 27 to markedly reduce ACh-evoked hyperpolarization of rat mesenteric smooth muscle, suggest that these observations of gap junction distribution truly reflect the requirement for functional gap junction channels in EDHF-type responses (Sandow *et al.*, 2002).

In addition to the intercellular spread of current, transfer of Ca^{2+} or IP_3 between vascular cells *via* gap junction channels may also be of physiological relevance owing to the importance of changes in $[\text{Ca}^{2+}]_i$ in the regulation of vascular tone. Indeed, IP_3 has been demonstrated to permeate gap junctions between porcine aortic endothelial cells (Carter *et al.*, 1996). The potential for myoendothelial Ca^{2+} signalling was described following the demonstration in hamster cheek pouch arterioles, that increases in smooth muscle cell $[\text{Ca}^{2+}]_i$ were accompanied by elevations in endothelial cell $[\text{Ca}^{2+}]_i$ and the synthesis of NO (Dora *et al.*, 1997). The subsequent demonstration in rat mesenteric arteries that, palmitoleic acid significantly reduced similarly evoked increases in endothelial cell $[\text{Ca}^{2+}]_i$ in response to smooth muscle activation with PE (Schuster *et al.*, 2001), suggested that gap junctions may be important in the communication of changes in $[\text{Ca}^{2+}]_i$ between the two cell types. Communicated changes in $[\text{Ca}^{2+}]_i$ from smooth muscle to endothelial cells are unlikely to result from the spread of current from depolarized smooth muscle cells as endothelial cell depolarisation *per se* would decrease the $[\text{Ca}^{2+}]_i$ as a result of decreased Ca^{2+} influx (Schilling, 1989). An alternative

explanation is the passage of Ca^{2+} or IP_3 down the concentration gradient from activated smooth muscle cells to adjacent endothelial cells, which may provide a mechanism for vasoconstriction-evoked NO release in rat mesenteric arteries (Dora *et al.*, 2000). However, due to extensive buffering of intracellular Ca^{2+} (Kasai & Petersen, 1994), intercellular passage of Ca^{2+} *per se* is unlikely.

1.3.2 Extracellular Diffusion

In addition to neurotransmitters released from perivascular nerves and the release of autacoids from endothelial cells (NO, EETs, anandamide, PGI_2 and K^+), extracellular diffusion of ATP between non-neuronal vascular cells may play a role in intercellular signalling within the artery wall.

ATP has an established role as an autocrine and paracrine signalling molecule in many cell types. These include pancreatic acini, ureter epithelium, renal epithelium, mast cells and astrocytes and can be released in response to hypotonicity, mechanical stimulation and/or Ca^{2+} agonists (Guthrie *et al.*, 1999; Knight *et al.*, 2002; Osipchuk & Cahalan, 1992; Schwiebert & Kishore, 2001; Sorensen & Novak, 2001). Upon release into the extracellular milieu, ATP and its metabolites are subject to rapid degradation by ecto-enzymes, limiting signalling by these mediators to autocrine and paracrine effects (Burnstock, 1997). As a ubiquitous intracellular constituent with multiple receptor subtypes on most cells, any cell is a potential source of releasable ATP and most cells can respond to its presence in the external environment.

P₂ receptors for ATP and ADP are present on smooth muscle and endothelial cells throughout the vascular system and play an important role in the regulation of vascular tone (Boarder & Hourani, 1998; Hill *et al.*, 2001). Activation of smooth muscle P₂ receptors, predominantly of the ligand-gated P2X₁ subtype evoke vasoconstriction (Boarder & Hourani, 1998; Hansen *et al.*, 1999; Ralevic & Burnstock, 1998), while G-protein-coupled P2Y receptors on the endothelium mediate vasodilatation by stimulating the release of NO and EDHF (Buvinic *et al.*, 2002; Malmstro *et al.*, 2002; Marrelli, 2001; Stanford *et al.*, 2001). The endogenous source of extracellular ATP activating smooth muscle P₂ receptors is principally sympathetic nerves (Boarder & Hourani, 1998; Ralevic & Burnstock, 1998), while activation of endothelial receptors most likely reflects release from platelets (Boarder & Hourani, 1998; Burnstock, 1997). However, other sources of endogenous ATP within the vasculature are the smooth muscle and endothelial cells themselves (Bodin *et al.*, 1991; Bodin & Burnstock, 1996; Bodin & Burnstock, 2001; Bodin & Burnstock, 1998; Burnstock, 1999; Pearson & Gordon, 1979; Shinozuka *et al.*, 1997). Mechanisms involving ATP binding cassette (ABC) proteins (Demolombe & Escande, 1996; Guidotti, 1996; Roman *et al.*, 2001; Schwiebert, 1999) and the release of vesicular stores of ATP by exocytosis (Knight *et al.*, 2002; Sorensen & Novak, 2001), have been implicated in ATP transport across the plasma membrane of non-neuronal cells. Vascular endothelial cells have been demonstrated to release ATP by the latter mechanism (Bodin & Burnstock, 2001). Within the artery wall, the close apposition of smooth muscle and endothelial cells raises the possibility that the release of ATP from either population of cells may act as an autocrine or paracrine signalling factor between and within each cell type.

1.4 Research aims

The experiments presented in this thesis were designed to further current knowledge on the role of gap junctions in the regulation of arterial diameter. More specifically, they were to investigate the requirement for these channels in the communication of changes in $[Ca^{2+}]_i$ between vascular endothelial cells and their role versus an extracellular mechanism in the transmission of signals between endothelial and smooth muscle cells in EDHF-type responses and vasoconstriction-evoked NO release.

CHAPTER TWO

Methods

2.1 Isometric tension recordings

The technique of wire myography for the measurement of isometric tension responses in small arteries (internal diameter 100-1000 μm) was first developed by Mulvany and Halpern (1997) Figure 2.1. The use of thin mounting wires ($\leq 40 \mu\text{m}$) allows arterial segments ($\leq 2000 \mu\text{m}$ in length) to be secured under tension without trauma to the tissue.

2.1.1 Preparation and mounting of arterial segments

Male Wistar rats (200–250 g) were killed by cervical dislocation and the mesenteric bed removed and placed in Krebs-Henseleit buffer (Appendix 1). Third-order branches of rat superior mesenteric artery (diameter 150-300 μm) were dissected and cleaned of surrounding fat and connective tissue under a light microscope (Stemi SV11, Carl Zeiss Ltd, Hertfordshire, U.K.). A small cut was made in the artery wall to allow the insertion of a 2 cm length of gold-plated tungsten wire (diameter 25 μm ; Goodfellow, Cambridge, U.K.). A 2 mm length of artery was then cut and transferred to the centre of the length of wire and placed in the bath of a Mulvany-Halpern wire myograph (Danish Myotechnology, 400A, JP Trading, Aarhus, Denmark) containing Krebs buffer maintained at 37 °C. The wire was then secured to the micrometer foot of the myograph with screws. Once anchored, a second 2 cm length of wire was passed through the lumen of the artery and screwed to the opposite foot of the myograph attached to the force transducer. The Krebs buffer was replaced every 30 minutes and gassed with 95% O_2 /5% CO_2 throughout the duration of the experiment.

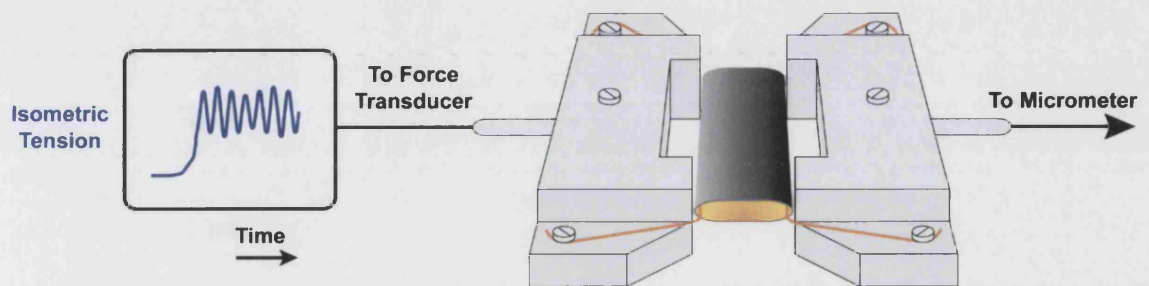


Figure 2.1 Diagrammatic representation of a Mulvany-Halpern wire myograph.

Gold-plated tungsten wires were inserted through the lumen of the arterial segment and clamped at each end to the feet of the myograph to ensure that responses were isometric. One foot was connected to the force transducer and the other to the micrometer. Diagram reproduced by kind permission of Dr. Kim Dora.

2.1.2 Normalization of arterial diameter

Following an equilibration period of 30 minutes, a passive-tension diameter curve was obtained for each arterial segment according to the methodology first proposed by Mulvany and Halpern (1977). By adjusting the micrometer, arterial segments were distended in a stepwise manner up to a maximum passive tension of 2 mN. For each increment, the micrometer setting and passive tension reading were noted and used to construct a curve of passive tension versus arterial diameter using a customised computer program developed by Dr. G. A. McPherson (Normalize; Monash University, Melbourne, Australia).

At constant transmural pressure, estimations of passive tension and arterial diameter can be made from the law of Laplace.

$$\text{Tension (T)} = \text{Pressure (P)} \times \text{Radius (r)}$$

Data calculated using this law and assuming a constant transmural pressure of 100 mmHg, were superimposed as an isobar over the previously determined passive tension/diameter exponential curve. The intersection point of the two data sets was taken as the internal diameter at which the artery would be when relaxed and under a transmural pressure of 100 mmHg. To obtain optimal responses to the application of contractile agonists, arterial diameter was set to 0.9 times this value (Mulvany & Halpern, 1977).

2.1.3 Experimental protocol

At the start of each experiment, endothelium viability was assessed as >95% relaxation to acetylcholine (1 μ M) in arteries contracted with phenylephrine (3 μ M). For the study of EDHF-type responses, arterial segments were pre-exposed to L-NAME (100 μ M) for 30 minutes. A cyclo-oxygenase inhibitor was not included in the bath, as it does not play a significant role as an endothelium-derived relaxation factor in this artery (Garland & McPherson, 1992). The same viability test was performed on arterial segments exposed to L-NAME.

Concentration-response curves were always obtained by the cumulative addition of increasing drug concentrations to the myograph bath. All relaxation responses were assessed by initially contracting arterial segments with phenylephrine. Variable levels of contraction ('low' < 70% of maximum or 'high' 70-100% of maximum) were achieved by titration with phenylephrine (0.1 – 10 μ M). In paired experiments, phenylephrine concentrations were altered to ensure the same level of contraction developed as in control arteries. Where contractions to the general NOS inhibitor, L-NAME (100 μ M; Alderton *et al.*, 2001), the soluble guanylate cyclase inhibitor, ODQ (10 μ M; Hobbs, 1997) and the nNOS selective inhibitor, propyl-L-arginine (PLA, 1 μ M; Zhang *et al.*, 1997) were investigated, arteries were initially contracted to \leq 25% of maximum contraction with either phenylephrine or KCl and allowed to plateau before the addition of L-NAME, ODQ or propyl-L-arginine. P_{2X1} receptor desensitization was achieved by incubation with α,β -methylene-ATP (α,β -MeATP, 10 μ M) for 10 minutes and confirmed by loss of contraction to the drug upon second exposure. To assess the effect of various agents on control responses, arterial segments were pre-incubated with

the drug for 30 minutes, with the exception of gap 27, which was present in the bath for three consecutive 40 minute periods. Arteries were denuded of endothelium by gentle rubbing of the luminal surface with a human hair. Removal of the endothelium was confirmed by the absence of relaxation to acetylcholine (1 μ M) in tissue contracted with phenylephrine (3 μ M). Maximum contraction was determined at the end of each experiment by the combined application of 65 mM K⁺ and 10 μ M phenylephrine.

Tension data was collected through a MacLab 2e interface (AD Instruments Ltd., Australia) using commercially available software (Chart; AD Instruments Ltd., Australia).

2.1.4 Analysis of data

All relaxation responses were expressed as the percentage reversal of agonist-induced contraction. Responses to constrictor agents were expressed as a percentage of maximum contraction. Values are presented as mean \pm S.E.M. Statistical analysis was performed using Student's t-test or one-way ANOVA followed by Bonferroni's post-test as appropriate. $P < 0.05$ was taken as statistically significant.

The maximum ability of an agonist to reverse phenylephrine-mediated contraction was indicated as the maximal relaxation response (R_{max}). EC_{50} values are presented as their negative logarithm (pD_2) and were calculated by fitting sigmoidal curves to log-concentration-response relationships (GraphPad Prism software; GraphPad Software Inc., San Diego, C.A, USA).

2.2 ATP assay

Quantitative detection of ATP by luciferase driven bioluminescence is an established technique exploiting the ATP dependency of luciferase-catalysed luciferin oxidation for the measurement of extremely low concentrations of ATP (McElroy & DeLuca, 1983).



The green light produced by this reaction has an emission maximum at 562 nm.

2.2.1 Sample preparation

Rat mesenteric arteries were obtained as previously described in section 2.1.1. Three ~1 cm length arterial segments were transferred to a polypropylene tube containing 100 μ l of MOPS buffer (Appendix 2) and stored on ice. This constituted one tissue sample. The process was repeated until enough tissue samples were obtained to perform all measurements of free ATP concentration in triplicate. Samples from a range of known ATP concentrations (log -12 to -6 M) were prepared and also stored on ice.

2.2.2 Protocol for measuring agonist-stimulated ATP release

ATP levels were determined using the ATP bioluminescence assay kit HS II (Roche) according to the manufacturer's protocol. Luminescence readings were measured using a tube-luminometer (Turner Designs, Model TD – 20/20). Briefly, luminescence readings obtained from the samples of known [ATP] were used to construct a standard

curve at the start of each experiment (Figure 2.2). Release of ATP from the tissue sample into the surrounding MOPS buffer was measured by transferring the 100 μ l sample of MOPS buffer to a cuvette with an equivalent volume of luciferase reagent. The cuvette was then inserted into the luminometer and a reading of luminescence obtained, from which the free ATP concentration could be calculated using the standard curve. To examine the effects of phenylephrine (10 μ M) or acetylcholine (10 μ M) stimulation on ATP release from the tissue, a minimal volume of either agonist was added to the tissue sample and incubated for 10 minutes. Subsequently, the 100 μ l sample of MOPS buffer was extracted and its ATP content measured. ATP measurements were repeated for the addition of both agonists to 100 μ l samples of MOPS buffer in the absence of tissue. An equivalent volume of MOPS buffer to that added for both agonists was added to separate tissue samples as vehicle controls. The effect of glibenclamide (10 μ M) and the vesicle fusion inhibitor, N-ethylmaleimide (1 μ M) on phenylephrine and acetylcholine evoked ATP release was determined by pre-incubation with these agents for 20 minutes prior to stimulation with either agonist. An equivalent volume of DMSO to that added for glibenclamide, was included prior to agonist stimulation in separate tissue samples as a vehicle control.

2.2.3 Analysis of data

Unknown concentrations of ATP were calculated by fitting a sigmoidal curve to the log-log concentration-bioluminescence relationship (GraphPad Prism software). Levels of free ATP from unknown samples were expressed as pmol/l. Values are presented as mean \pm S.E.M. Statistical comparisons were made using one-way ANOVA followed by Bonferroni's post-test. $P < 0.05$ was taken as statistically significant.

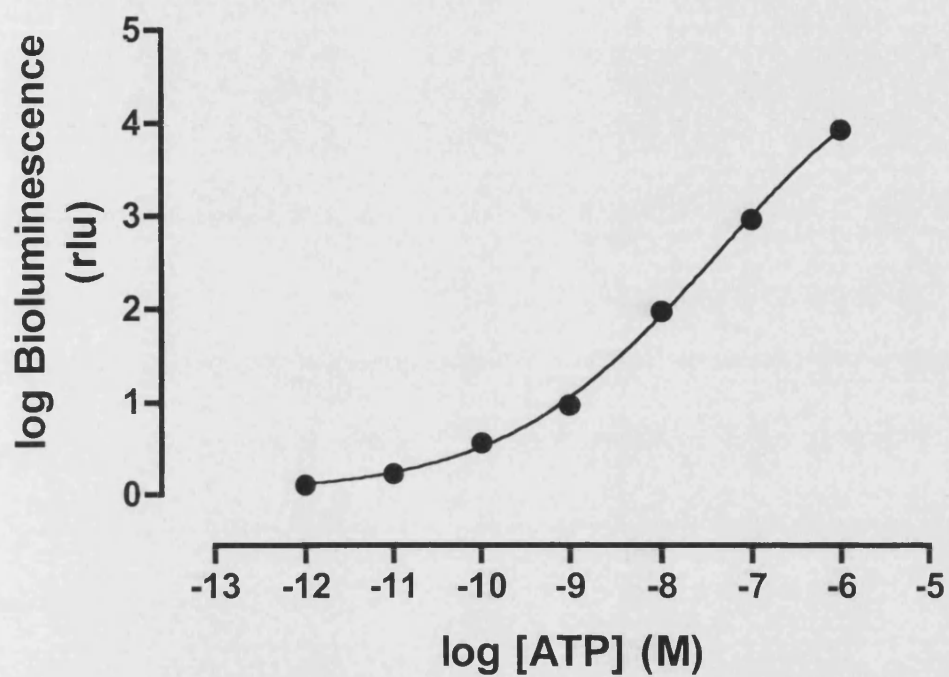


Figure 2.2 Typical ATP standard curve

An ATP standard curve was produced at the start of each experiment from known concentrations of ATP. Rlu indicates units of relative luminescence.

2.3 Cell isolation and culture

The isolation of vascular smooth muscle and endothelial cells enabled calcium imaging and fluorescent labelling experiments to be performed on individual cell types.

2.3.1 Isolation of mesenteric cells

Rat mesenteric arteries were obtained as previously described in section 2.1.1, cut open longitudinally and placed into low Ca^{2+} PSS (Appendix 3) containing 1mg/ml papain, 1mg/ml dithiothreitol (DTT) and 1mg/ml bovine serum albumin. For the isolation of endothelial cells, arteries were incubated in the enzyme mixture for 10 minutes at room temperature and a further 7 minutes at 37°C in a shaking incubator. Smooth muscle cell isolation followed the same protocol but required an additional 8 minutes at 37°C. Following enzyme digestion, arteries were washed several times in MOPS buffer before being transferred to a polypropylene tube containing 300 μl Ca^{2+} -free PSS (Appendix 4). Trituration in Ca^{2+} -free PSS with a fire polished glass pipette dispersed individual and groups of cells from the digested tissue. For smooth muscle cell isolation, the tissue was subjected to several trituration steps, each time being transferred to a fresh 300 μl sample of Ca^{2+} -free PSS.

2.3.2 Isolation of aortic cells

Male Wistar rats (200–250 g) were killed by cervical dislocation. Thoracic and abdominal sections of aorta were removed and placed in Krebs buffer. Vessels were cleaned of adherent fat and connective tissue, cut open longitudinally and then into

smaller strips. Isolated endothelial and smooth muscle cells were obtained as described in section 2.3.1.

2.3.3 Cell culture

Aortic endothelial or smooth muscle cells isolated by the method described in section 2.3.2 were plated onto 24mm diameter coverglasses. Coverglasses upon which endothelial cells were to be plated were first coated with endothelial cell attachment factor (ECAAF), a solution containing factors from iron-supplemented calf serum associated with endothelial cell attachment and spreading. Once the cells had adhered, the coverglasses were transferred to 35mm diameter petri dishes to which 3 ml of Dulbeccos modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100µg/ml gentamycin and 2mM L-glutamine was added. The petri dishes containing the cells were then placed in a humidified incubator maintained at 37°C. The media was replaced every 24 hours and the cells used in experiments after 72-96 hours.

2.4 Fluorescent labelling

2.4.1 Cell and tissue preparation

Mesenteric endothelial and smooth muscle cells were isolated by the method described in section 2.3.1. Drops of triturate containing cells dispersed from the digested tissue were placed onto a 24mm diameter coverglass within a thermostatically controlled perfusion chamber maintained at 37°C. Once adhered to the coverglass, cells were selected for use according to their morphology in Ca^{2+} -free solution, smooth muscle cells appearing large and elongated and endothelial cells smaller and round (Figure 2.3). Cells of interest were positioned within the field of view and extracellular Ca^{2+} returned by superfusion with MOPS buffer.

Cultured aortic endothelial and smooth muscle cells were obtained by the methods described in sections 2.3.2 and 2.3.3. The culture medium was removed and the coverglasses rinsed several times with MOPS buffer before being transferred to a thermostatically controlled perfusion chamber containing MOPS buffer maintained at 37 °C.

Thoracic sections of aorta were obtained by the methods described in section 2.3.2 to fluorescently label the intact arteries.

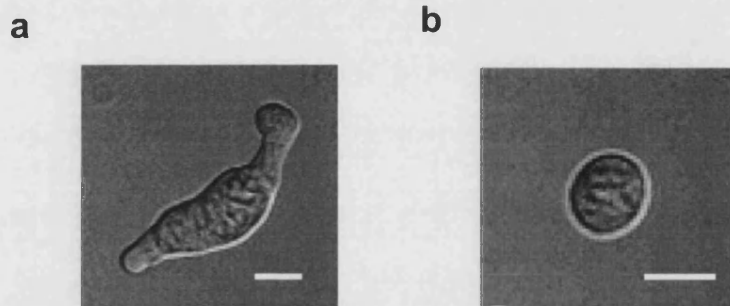


Figure 2.3 Isolated mesenteric smooth muscle and endothelial cells in Ca^{2+} -free PSS.

Examples of isolated mesenteric smooth muscle (a) and endothelial cells (b) identified by their distinct morphology in Ca^{2+} -free solution. Endothelial cells appeared small and round while smooth muscle cells were larger and elongated. Bar = 20 μm .

2.4.2 Labelling of aortic endothelial cells

To optimise the cell isolation protocol, preliminary experiments were performed in which endothelial cells in culture were identified by their unique ability to accumulate acetylated-low density lipoprotein (Ac-LDL, Voyta *et al.*, 1984) and by the presence of adhesive glycoprotein von Willebrand factor (vWF), not present in vascular smooth muscle. The fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) was attached to Ac-LDL to enable detection by fluorescence microscopy.

2.4.2.1 Uptake of DiI-Ac-LDL

For the labelling of cultured cells, the culture media was replaced by fresh media containing DiI-Ac-LDL (10 µg/ml, peak Abs 554 nm, peak Em 571 nm) and the cells returned to the incubator for a further 4 hours. The cells were subsequently washed with probe-free media, rinsed with MOPS buffer and transferred to a thermostatically controlled chamber containing MOPS buffer maintained at 37 °C.

Intact sections of aorta were placed abluminal surface down onto a 24 mm diameter coverglass. The coverglass was then transferred to a 35 mm diameter petri dish to which 3 ml of DMEM containing DiI-Ac-LDL (10µg/ml) was added before being placed in a humidified incubator maintained at 37°C for 24 hours. The tissue was then washed with probe-free media and rinsed with MOPS buffer before being transferred to a thermostatically controlled chamber containing MOPS buffer maintained at 37 °C. The cell permeant nucleic acid stain SYTO-16 (100 nM, peak Abs 488 nm, peak Em 518

nm) was added to the MOPS buffer for 2 hours prior to viewing to identify endothelial and smooth muscle cell layers by shape and orientation. Barrier filters BA560IF and BA505-525 were used for DiI-Ac-LDL and SYTO-16 detection respectively.

2.4.2.2 Immunostaining

Cultured cells were rinsed several times with MOPS buffer and fixed in 2% paraformaldehyde in PBS for 20 min. Once fixed, cells were permeabilized by incubation in phosphate buffered saline (PBS) containing 0.2% Triton X-100 and 10% normal goat serum (NGS) for 20 min and subsequently incubated for 2 hours with a monoclonal primary antibody raised in rabbit against vWF (diluted 1:300 in PBS containing 1% NGS and 0.025% sodium azide). After washing in PBS, cells were incubated with a FITC-labelled secondary antibody (goat anti-rabbit IgG, peak Abs 494 nm, peak Em 519 nm) for 1 hour (diluted 1:300 in PBS containing 1% NGS and 0.025% sodium azide). In control experiments, cells were incubated in 1% NGS and 0.025% sodium azide in the absence of primary antibody. After washing in PBS, coverglasses were mounted in Vectashield® and allowed to dry. Barrier filter BA505IF was used for FITC detection.

2.4.3 Quinacrine and mitofluor labelling

Freshly isolated mesenteric smooth muscle and endothelial cells were incubated with the fluorescent ATP marker, quinacrine (100nM, peak Abs 476 nm, peak Em 490-540 nm, Bodin & Burnstock, 2001; Mitchell *et al.*, 1998; Sorensen & Novak, 2001), for 1 hour. After 15 minutes of the 1 hour period, the fluorescent mitochondrial marker,

mitofluor (100nM, peak Abs 589 nm, peak Em 630 nm), was added and remained in the bath solution for the following 45 minutes. Cells were then rinsed by superfusion with MOPS buffer. Barrier filters BA505-525 and BA610IF were used for quinacrine and mitofluor detection respectively.

2.4.4 Dye transfer

The cell-impermeant fluorescent dye, lucifer yellow (45 mM, peak Abs 426 nm, peak Em 531nm) was introduced into a number of cells by scrape loading (Domenighetti *et al.*, 1998). A scalpel blade was gently drawn across the cultured endothelial cells in the presence of lucifer yellow. After a loading period of two minutes, the cells were washed several times in MOPS buffer. Barrier filter BA505IF was used for Lucifer yellow detection.

2.4.5 Analysis of data

All images were collected and analysed using an inverted confocal laser scanning microscope (Olympus Fluoview 500 laser scanner attached to an Olympus IX70 inverted microscope, Fluoview software version 3.1) equipped with two lasers: argon (488 nm, blue) and helium-neon (543 nm, green). A 40x water immersion objective (Olympus, numerical aperture 1.3) was used in all experiments.

2.5 Calcium imaging

Freshly isolated and cultured cells for use in calcium imaging experiments were loaded with the fluorescent Ca^{2+} indicator fluo-4 AM (50 μM , peak Abs 494 nm, peak Em 516 nm) for 30 minutes under static conditions at room temperature and rinsed by superfusion with MOPS buffer. Barrier filter BA505IF was used for fluo-4 detection.

2.5.1 Cell preparation

Mesenteric endothelial and smooth muscle cells were isolated by the method described in section 2.3.1. Drops of triturate containing cells dispersed from the digested tissue were placed onto a 24mm diameter coverglass within a thermostatically controlled perfusion chamber maintained at 37°C. Once adhered to the coverglass, cells were selected for use according to their morphology in Ca^{2+} -free solution, smooth muscle cells appearing larger and elongated and endothelial cells smaller and round (Figure 2.3). Cells of interest were positioned within the field of view and extracellular Ca^{2+} returned by superfusion with MOPS buffer.

Cultured aortic endothelial cells were obtained by the methods described in sections 2.3.2 and 2.3.3. The culture medium was removed and the coverglasses rinsed several times with MOPS buffer before being transferred to a thermostatically controlled perfusion chamber containing MOPS buffer maintained at 37 °C.

2.5.2 Agonist stimulation

Changes in $[Ca^{2+}]_i$ in response to agonist stimulation were examined by adding the required volume of agonist under conditions of no flow to a predetermined volume of MOPS buffer in which the cells were bathed. Cells were exposed to agonists for ~ 90 seconds before being washed with MOPS buffer by resuming superfusion. A period of at least 10 minutes was allowed between agonist additions. When required, cells were exposed to palmitoleic acid (50 μ M) for 1 hour prior to agonist stimulation.

2.5.3 Mechanical stimulation

Changes in $[Ca^{2+}]_i$ in response to a mechanical stimulus were investigated under conditions of flow (2ml/min) in either MOPS buffer or Ca^{2+} -free PSS. A mechanical stimulus was applied to a single cell by temporary perturbation of the membrane with a pulled glass micropipette controlled by a manual micromanipulator (LBM-7, Scientifica, Herts, U.K., Figure 2.4). Successful perturbation of the membrane without rupture was indicated by the retention of fluo-4 and recovery to baseline fluorescence following stimulus application. A period of at least 10 minutes was allowed between stimulations. In the relevant experiments, cells were exposed to cyclopiazonic acid (CPA, 10 μ M) and U-73122 (10 μ M) for 30 minutes prior to mechanical stimulation. A 1 hour exposure period was used for palmitoleic acid (50 μ M).

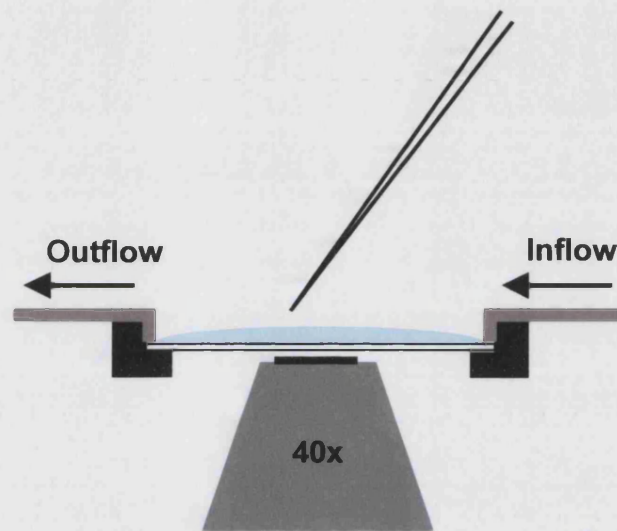


Figure 2.4 Diagrammatic representation of the technique used to apply a mechanical stimulus to a single cell in culture.

Coverglasses of cultured cells were mounted in a thermostatically controlled (37 °C) superfusion chamber and visualized with a 40x water immersion objective. A mechanical stimulus was applied to a single cell with a pulled glass micropipette

2.5.4 Analysis of data

All images were collected and analysed using an inverted confocal laser scanning microscope (Olympus Fluoview 500 laser scanner attached to an Olympus IX70 inverted microscope, Fluoview software version 3.1) equipped with an argon-ion laser (488 nm, blue) and a 40x water immersion objective (Olympus, numerical aperture 1.3). Images of fluorescence intensity (505 nm) were collected at 1.04 Hz or 500 Hz. Responses to agonist or mechanical stimulation were expressed either as a peak percentage change in fluorescence intensity, a percentage change in fluorescence intensity over time or as the number of cells responding with an increase in fluorescence intensity. Values are presented either as raw data or means \pm S.E.M as indicated. Statistical analysis was performed using Students' paired t-test or one-way ANOVA followed by Bonferroni's post test as appropriate. $P < 0.05$ was taken as statistically significant.

2.6 Drugs and chemicals

The following agents were used: acetylcholine chloride, adenosine 5'-triphosphate, ATP bioluminescence assay kit HS II, bovine serum albumin, bradykinin, carbenoxolone, cyclopiazonic acid, (DMSO), diethylamine NONOate, DiI-Ac-LDL, dimethylsulphoxide, dithiothreitol, Dulbecco's modified Eagle medium, N-ethylmaleimide (EtOH), foetal bovine serum, fluo-4 (DMSO), Gap 27 (sequence SRPTEKTIFII), gentamycin, glibenclamide (DMSO), L-glutamine, iberiotoxin, indomethacin (2% sodium bicarbonate), levcromakalim (DMSO), lucifer yellow, α,β -methyleneadenosine 5'-triphosphate, mitofluor (DMSO), N^ω-nitro-L-arginine methyl ester, normal goat serum, ODQ, palmitoleic acid, papain, phenylephrine hydrochloride, phosphate buffered saline, pluronic, potassium chloride, N^ω-propyl-L-arginine, quinacrine, sodium azide, syto-16 (DMSO), triton X-100, and U-73122. All compounds used were from Sigma Chemical Company (Poole, UK) except pluronic, DiI-Ac-LDL, mitofluor and fluo-4-AM (special packaging) which were purchased from Molecular Probes (Leiden, The Netherlands), N^ω-propyl-L-arginine from Tocris (Avonmouth, U.K), Gap 27 from Severn Biotech (Kidderminster, U.K), dithiothreitol from Melford laboratories (Ipswich, U.K), diethylamine NONOate from Calbiochem (Nottingham, U.K), foetal bovine serum, Dulbecco's modified Eagle medium and gentamycin from Gibco (Paisley, U.K.), U-73122 from Biomol (Exeter, U.K.), iberiotoxin from Latoxan (Valence, France) and ATP bioluminescence assay kit HS II from Roche (Sussex, U.K). Antibodies were purchased from Sigma. All drugs were dissolved in either distilled water, Krebs or MOPS buffer except where indicated otherwise. Fluo-4 AM was initially dissolved in DMSO and diluted to the appropriate concentration in MOPS

buffer. Fluo-4 solutions also contained pluronic (0.5%, Molecular Probes) and were filtered with a 0.22 μm syringe-driven filter unit.

CHAPTER THREE

Intercellular calcium signalling via gap junction channels in rat aortic endothelial cells

3.1 Introduction

Increases in intracellular Ca^{2+} within vascular smooth muscle and endothelial cells mediate changes in arterial diameter by evoking vasoconstriction and vasodilatation respectively. The mechanisms by which these two cell types may communicate changes in $[\text{Ca}^{2+}]_i$ are therefore of considerable importance to the regulation of changes in vascular tone.

Intercellular Ca^{2+} signalling has been described in many cell types and is believed to play an important role in the co-ordination of cellular responses. In ciliated airway epithelial cells, an acceleration in ciliary beat frequency was observed upon mechanical stimulation of a single cell, which also occurred in neighbouring non-stimulated cells (Sanderson *et al.*, 1988). Ca^{2+} imaging revealed this response to be controlled by an intercellular Ca^{2+} wave and so demonstrated intercellular Ca^{2+} signalling to provide airway epithelial cells with a physiological mechanism for cellular co-operation (Sanderson *et al.*, 1990). Hepatocytes have also been shown to exhibit synchronous multicellular changes in $[\text{Ca}^{2+}]_i$ which are believed to be important for a co-ordinated glycogenolytic response induced by hormonal stimulation (Saez *et al.*, 1989). In the majority of cell types studied, gap junctions are thought to provide the route whereby changes in $[\text{Ca}^{2+}]_i$ can be communicated to neighbouring cells, however a number of studies have provided evidence for an extracellular route of intercellular Ca^{2+} signalling mediated by diffusion of ATP between cells (Guthrie *et al.*, 1999; Jorgensen *et al.*, 1997; Osipchuk & Cahalan, 1992).

Injection of hepatocytes with Ca^{2+} and IP_3 provided the first evidence that these second messengers can be transmitted from cell to cell through gap junctions (Saez *et al.*, 1989). However, due to extensive buffering of intracellular Ca^{2+} (Kasai & Petersen, 1994), most experimental evidence is now supportive of a mechanism of intercellular Ca^{2+} signalling dependent upon the intercellular diffusion of IP_3 . Unlike Ca^{2+} , IP_3 is not subject to extensive intracellular buffering, which is reflected in the maximum length constants for diffusion of these messengers of $4\mu\text{m}$ and $17\mu\text{m}$ respectively (Kasai & Petersen, 1994). Support for the gap junctional transfer of IP_3 in the mediation of spreading Ca^{2+} responses between cells, has come from studies in airway epithelia (Sanderson *et al.*, 1990) and pancreatic acini (Yule *et al.*, 1996) in which microinjection of IP_3 into a single cell evoked a communicated Ca^{2+} response. The requirement of phospholipase C activity for the propagation of intercellular Ca^{2+} waves between astrocytes (Venance *et al.*, 1997) and airway epithelial cells (Hansen *et al.*, 1995) in addition to the ability of IP_3 receptor blockade with heparin to inhibit the propagation of mechanically induced Ca^{2+} waves in airway epithelial cells (Boitano *et al.*, 1992), is in further support of a role IP_3 in intercellular Ca^{2+} signalling.

One of the first demonstrations that endothelial cells have the capacity to exchange dye tracers and small molecules was shown in cultured cells from bovine aorta and umbilical vein (Larson & Sheridan, 1982). The ability of endothelial cells to communicate changes in $[\text{Ca}^{2+}]_i$ between cells was subsequently shown in cultured cells from bovine aorta in which a diffusible substance other than Ca^{2+} was thought to mediate the response (Demer *et al.*, 1993). The subsequent demonstration that caged IP_3 can permeate porcine aortic endothelial cell gap junctions (Carter *et al.*, 1996) suggested that intercellular diffusion of this second messenger may underlie

intercellular Ca^{2+} signalling in this cell type. Co-ordinated changes in $[\text{Ca}^{2+}]_i$ within the endothelium may be of physiological relevance in the optimisation of EDRF-mediated changes in vascular tone. The present study investigates the ability of endothelial cells of the rat aorta to communicate increases in $[\text{Ca}^{2+}]_i$ between cells and addresses the potential role for gap junctions in this response.

3.2 Methods

Rat aortic endothelial cells were isolated and cultured as described in sections 2.3.2 and 2.3.3. Sections 2.4.1, 2.4.2, 2.4.4 and 2.4.5 describe how the cultured cells were prepared for use, labelled with fluorescent probes and imaged by confocal microscopy. The methodology employed to monitor changes in $[Ca^{2+}]_i$ in response to agonist or mechanical stimulation is described in section 2.5.

3.3 Results

3.3.1 DiI-Ac-LDL and vWF labelling of aortic endothelial and smooth muscle cells

The fluorescent endothelial marker DiI-Ac-LDL (10 $\mu\text{g/ml}$), labelled $98.2 \pm 0.2\%$ ($n = 5$) of the cultured isolated rat aortic cells with which it was incubated (Figure 3.1a and b). Uptake of DiI-Ac-LDL by intact sections of intact aorta was found to be restricted to the endothelial cell layer (Figure 3.1c, d, e and f, $n = 3$). Similarly, cultured cells isolated by the endothelial cell isolation procedure were labelled with antibodies to vWF (Figure 3.2a, $n = 2$), while cells isolated by the smooth muscle cell isolation procedure were not (Figure 3.2b, $n = 2$). No fluorescence was observed in control experiments in the absence of primary antibody ($n = 2$). n indicates the number of animals from which cells were obtained.

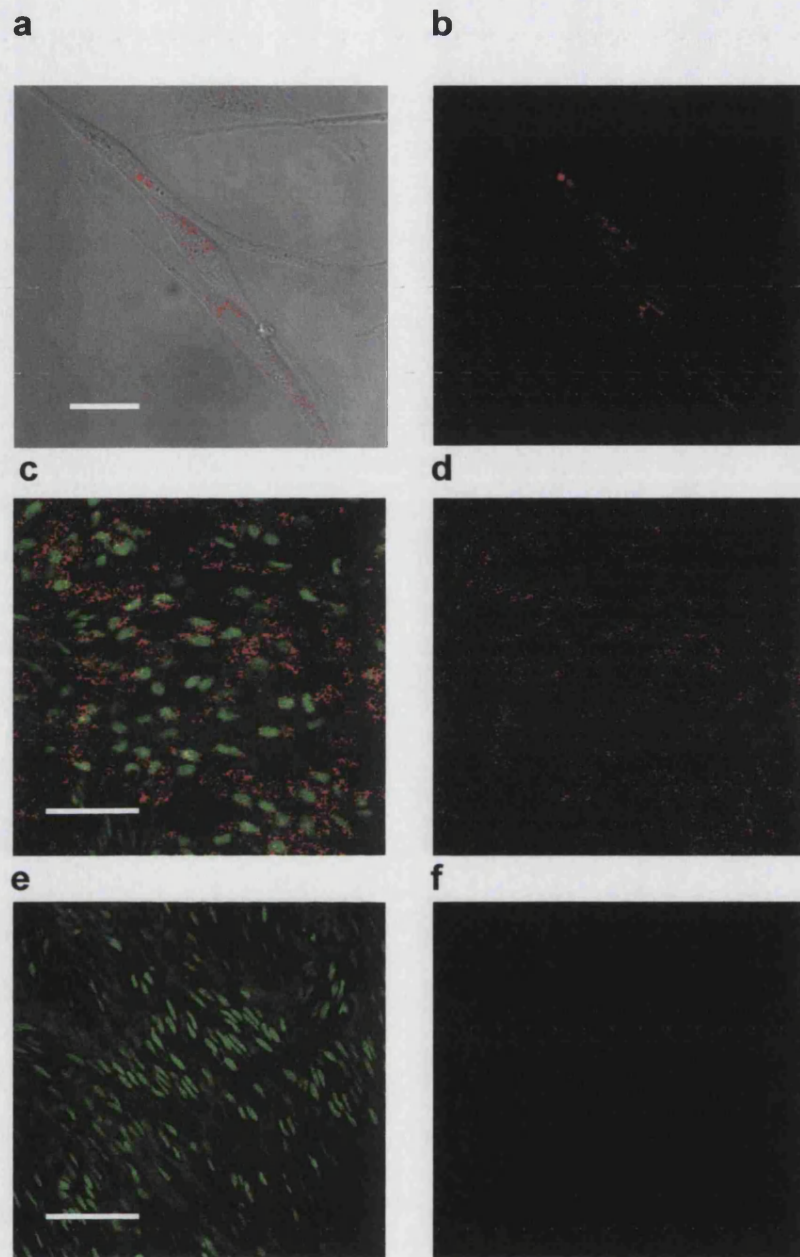


Figure 3.1 Selective uptake of DiI-Ac-LDL by aortic endothelial cells.

Representative images. a) Bright-field/fluorescence image overlay of 3 endothelial cells in culture labelled with DiI-Ac-LDL (10 $\mu\text{g/ml}$, bar = 40 μm). b) Fluorescence image of a. c) Fluorescence overlay of a confocal section through the endothelial layer of intact aorta following incubation with SYTO-16 (100 nM, nuclear stain, green, barrier filter BA505-525) and DiI-Ac-LDL (red, bar = 50 μm , barrier filter BA560IF). d) Fluorescence emission shown in c through barrier filter BA560IF only. e) Fluorescence overlay of a confocal section through one smooth muscle cell layer of intact aorta following incubation with SYTO-16 and DiI-Ac-LDL (bar = 50 μm , barrier filters BA505-525 and BA560IF). Bar = 50 μm . f) Fluorescence emission shown in e through barrier filter BA560IF only. Image settings for e and f are identical to those for c and d.

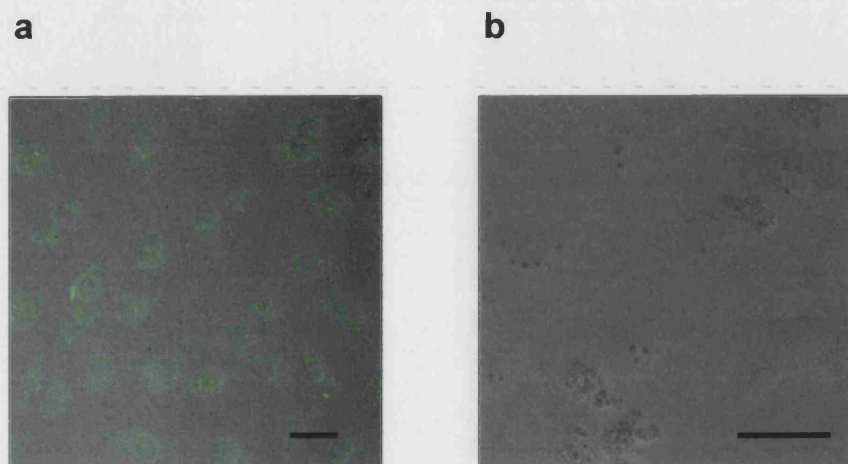


Figure 3.2 Cultured rat aortic endothelial and smooth muscle cells incubated with FITC-labelled antibodies to vWF.

Representative images. Bright-field/fluorescence image overlays. Endothelial cells in culture (a) were clearly labelled with the antibody, while smooth muscle cells (b) exhibited only background fluorescence at the same filter setting used for a (BA505IF). Bars = 50 μ m.

3.3.2 Dye Transfer

Lucifer yellow dye (45 mM) introduced into cultured rat aortic endothelial cells damaged by the scrape loading procedure (see section 2.4.4), diffused to non-damaged cells adjacent to the scrape line (Figure 3.3, $n = 5$). n indicates the number of animals from which cells were obtained.

3.3.3 Ca^{2+} responses to bradykinin

Fluo-4 (50 μM) loaded rat aortic endothelial cells in culture responded with an increase in fluorescence intensity to stimulation with bradykinin (50 nM, peak response $171 \pm 41\%$, Figure 3.4, $n = 6$, 24 cells). The presence of the gap junction uncoupler palmitoleic acid (PA, 50 μM , Burt *et al.*, 1991) was without effect on the bradykinin evoked increase in $[\text{Ca}^{2+}]_i$ (peak response $161 \pm 20\%$, Figure 3.4, $P > 0.05$, $n = 6$, 24 cells).

3.3.4 Ca^{2+} responses to mechanical stimulation

Fluo-4 (50 μM) loaded rat aortic endothelial cells in culture responded to mechanical stimulation of a single cell with a rapid rise in fluo-4 fluorescence which spread to an average of 4.8 ± 1.2 non-stimulated neighbouring cells (Figure 3.5, $n = 5$). A delay of 2.1 ± 0.5 s was observed between the onset of a mechanically induced rise in $[\text{Ca}^{2+}]_i$ within a stimulated cell and initiation of the rise in $[\text{Ca}^{2+}]_i$ within an adjacent cell (Figure 3.6, $n = 3$). Responding cells were defined as those exhibiting a $\geq 5\%$ or greater rise in basal fluorescence intensity.

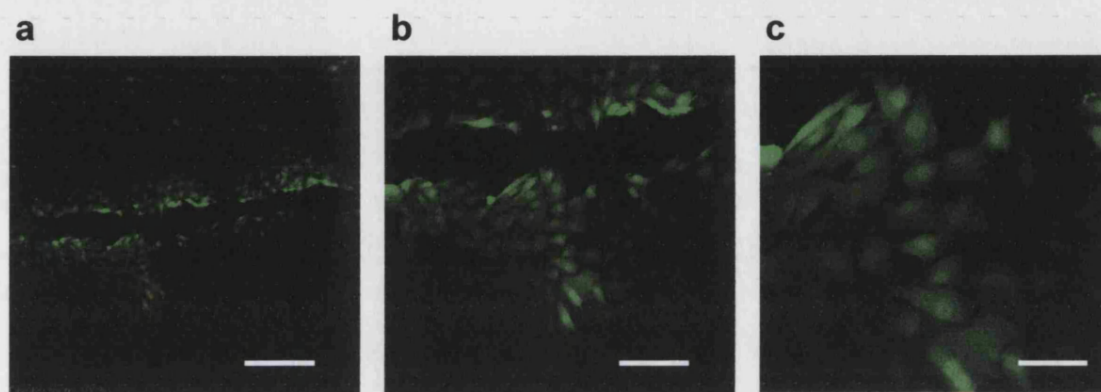


Figure 3.3 Dye transfer between cultured rat aortic endothelial cells.

Representative images. Fluorescence images of rat aortic endothelial cells in culture, following scrape loading with Lucifer yellow (45 mM, barrier filter BA505IF). Transfer of lucifer yellow away from cells of the scrape line becomes clearly visible upon greater magnification. a) 10x magnification, bar = 100 μm . b) 20x magnification, bar = 50 μm . c) 40x magnification, bar = 25 μm .

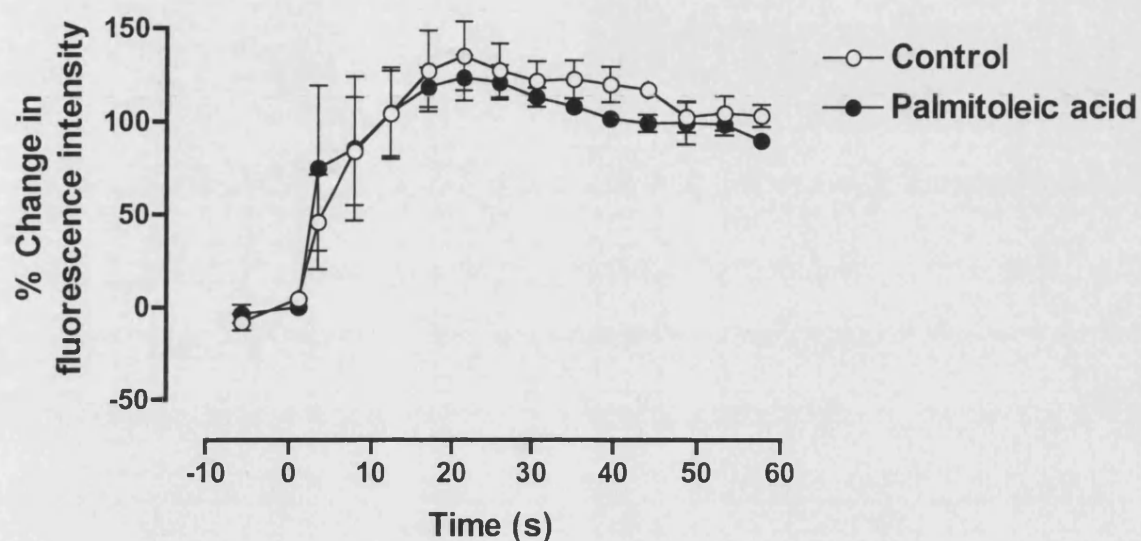


Figure 3.4 Effect of PA on bradykinin-evoked changes in $[Ca^{2+}]_i$ in cultured rat aortic endothelial cells.

Cultured endothelial cells loaded with Fluo-4 (50 μ M) responded with an increase in fluorescence intensity to stimulation with bradykinin (50 nM, control, peak response $171 \pm 41\%$, $n = 6$, 24 cells). Responses to bradykinin were unaffected by the presence of PA (50 μ M, peak response $161 \pm 20\%$, $n = 6$, 24 cells). Values are mean \pm S.E.M from n animals.

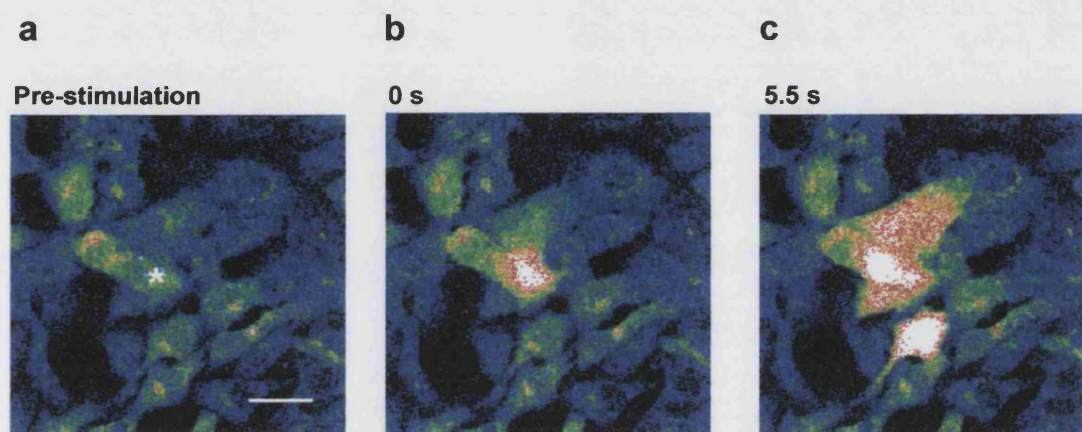


Figure 3.5 Calcium responses by cultured rat aortic endothelial cells to mechanical stimulation.

Time-sequence pseudocolour fluorescence images of changes in fluo-4 fluorescence upon mechanical stimulation of a single cell (representative response). The stimulated cell (*) and a number of adjacent cells responded with an increase in fluorescence. a) Pre-stimulation; b) at time of stimulation; c) 5.5 seconds after stimulation. Bar = 50 μm .

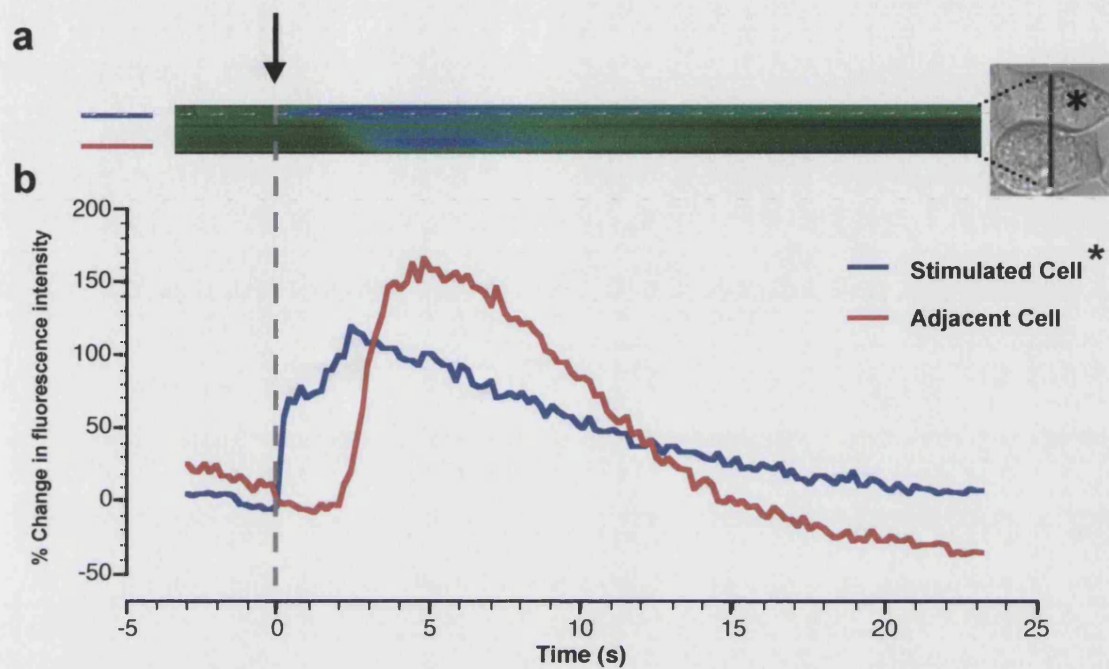


Figure 3.6 Time course of the spreading calcium response.

A representative response of the time course of changes in fluo-4 fluorescence within a mechanically stimulated cell (*, blue) and a single adjacent cell (red). a) A scanning line was positioned over two adjacent cells and fluorescence intensity sampled at 500 Hz. Each line scan (y-axis) is displayed over time (x-axis). b) Plot of intensity over time at position indicated by coloured bars in (A). Arrow indicates time of mechanical stimulation. The delay between the onset of the Ca^{2+} rise within the stimulated cell and the spread of the response to the adjacent cell can be seen in both a and b. Time scale identical in a and b.

3.3.5 Effect of PA on the spreading Ca^{2+} response induced by mechanical stimulation

The presence of the gap junction uncoupling agent, PA (50 μM), inhibited non-stimulated cells from responding with an increase in $[\text{Ca}^{2+}]_i$ to mechanical stimulation of a single neighbouring cell (0.8 ± 0.4 cells, Figure 3.7a, $P < 0.05$, $n = 5$). The inhibitory effect of palmitoleic acid was not attributable to a non-specific action of the gap junction uncoupling agent upon Ca^{2+} mobilisation within the stimulated cell, as the increase in fluorescence intensity within the stimulated cell was unaltered from that of control ($195 \pm 41\%$, Figure 3.7b, $P > 0.05$, $n = 5$).

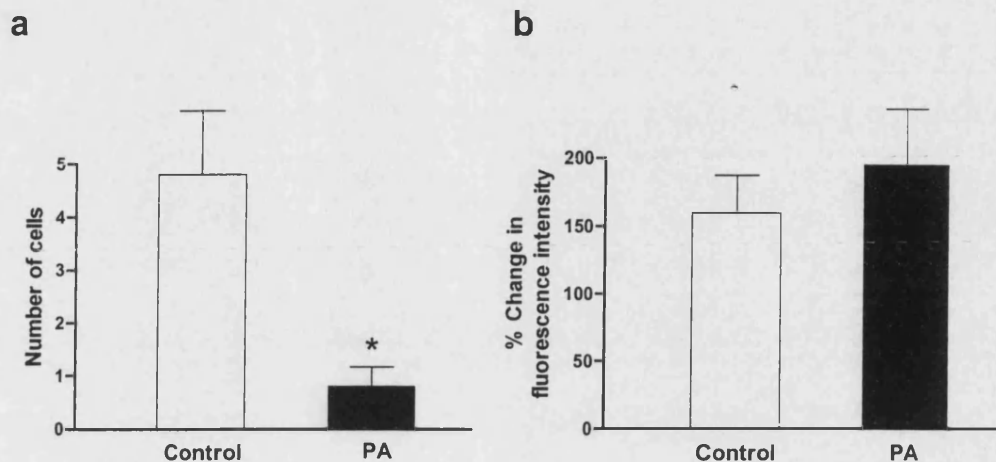


Figure 3.7 Effect of PA on mechanical stimulation induced calcium responses in cultured rat aortic endothelial cells.

Effect of PA (50 μ M) on a) the number of cells participating in the spreading Ca^{2+} response and b) the magnitude of the Ca^{2+} response within the stimulated cell. Palmitoleic acid restricted the stimulated rise in fluo-4 fluorescence to 0.8 ± 0.4 adjacent non-stimulated cells ($n = 5$). This differed significantly from control which spread to 4.8 ± 1.2 adjacent cells ($P < 0.05$, $n = 5$). The magnitude of the Ca^{2+} response within the stimulated cell was unaltered between control ($160 \pm 28\%$, $n = 5$) and PA ($195 \pm 41\%$, $n = 5$) treated groups ($P > 0.05$). Values are means \pm S.E.M from n animals. Analysis by Students' unpaired t-test; *, $P < 0.05$.

3.3.6 Effect of Ca^{2+} -free conditions on the spreading Ca^{2+} response induced by mechanical stimulation

In the absence of extracellular Ca^{2+} , the number of non-stimulated cells responding with an increase in $[\text{Ca}^{2+}]_i$ to mechanical stimulation of a single neighbouring cell, was unaltered from that of control (4.0 ± 0.7 cells, Figure 3.8a, $P > 0.05$, $n = 5$). However, no increase in fluo-4 fluorescence was observed within the stimulated cell ($-10 \pm 13\%$, Figure 3.8b, $P < 0.05$, $n = 5$). In 3 out of 5 cases a decrease in fluorescence was observed.

3.3.7 Effect of cyclopiazonic acid (CPA) on the spreading Ca^{2+} response induced by mechanical stimulation

Incubation with the Ca^{2+} -ATPase inhibitor CPA ($10 \mu\text{M}$), blocked the mechanical stimulation-induced rise in $[\text{Ca}^{2+}]_i$ within neighbouring non-stimulated cells (0.4 ± 0.3 cells, Figure 3.9a, $P < 0.05$, $n = 11$), but was without effect on the magnitude of the increase in fluo-4 fluorescence within the mechanically stimulated cell ($147 \pm 19\%$, Figure 3.9b, $P > 0.05$, $n = 11$).

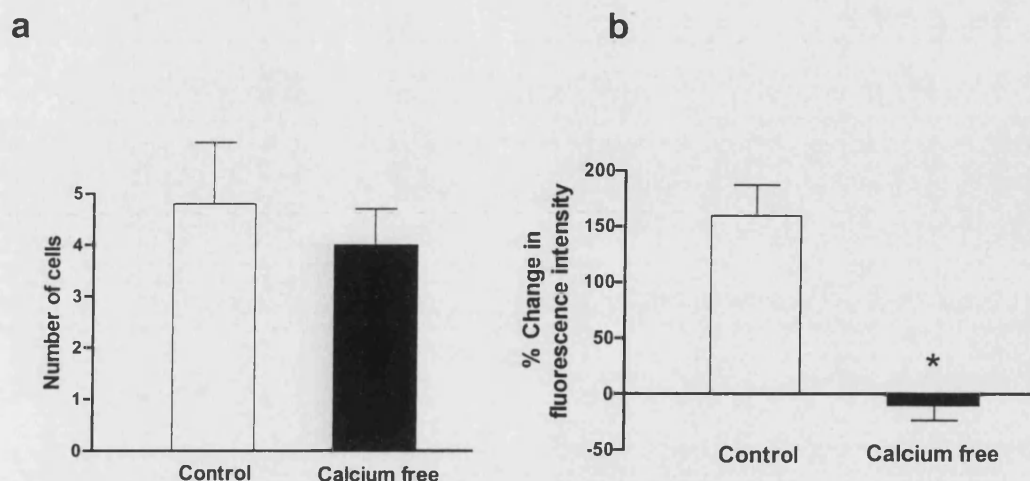


Figure 3.8 Effect of Ca^{2+} -free conditions on mechanical stimulation induced calcium responses in cultured rat aortic endothelial cells.

Effect of Ca^{2+} -free superfusion solution on a) the number of cells participating in the spreading Ca^{2+} response and b) the magnitude of the Ca^{2+} response within the stimulated cell. Ca^{2+} free solution was without effect on the number of non-stimulated cells in which a rise in fluo-4 fluorescence was evoked (4.8 ± 1.2 , $n = 5$ and 4.0 ± 0.7 , $n = 5$ respectively for control and Ca^{2+} -free conditions respectively, $P > 0.05$). However, in Ca^{2+} -free conditions a rise in fluo-4 fluorescence was not evoked in the stimulated cell ($160 \pm 28\%$, $n = 5$ and $-10 \pm 13\%$, $n = 5$, for control and Ca^{2+} -free conditions respectively, $P < 0.05$). Values are means \pm S.E.M from n animals. Analysis by Students' unpaired t-test; *, $P < 0.05$.

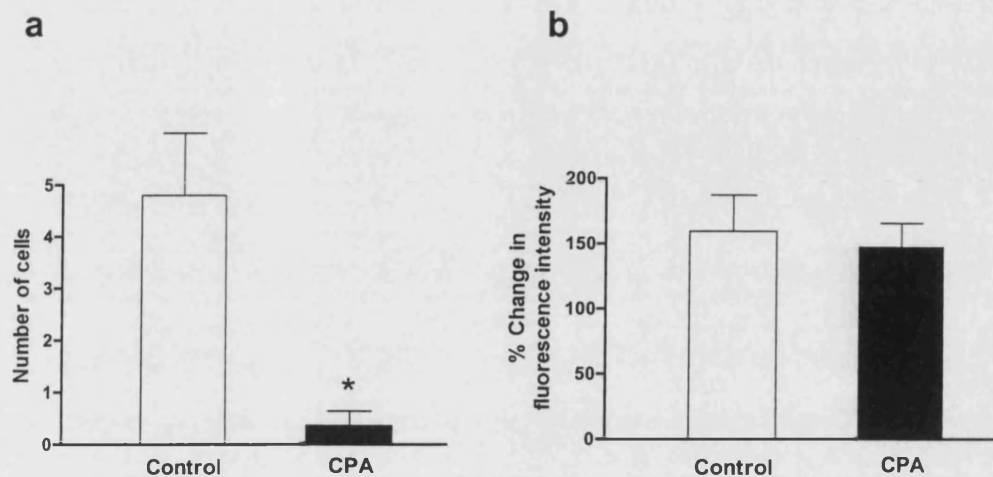


Figure 3.9 Effect of CPA on mechanical stimulation induced calcium responses in cultured rat aortic endothelial cells.

Effect of CPA (10 μ M) on a) the number of cells participating in the spreading Ca^{2+} response and b) the magnitude of the Ca^{2+} response within the stimulated cell. CPA restricted the stimulated rise in fluo-4 fluorescence to 0.4 ± 0.3 adjacent non-stimulated cells ($n = 11$). This differed significantly from control which spread to 4.8 ± 1.2 adjacent cells ($P < 0.05$, $n = 5$). The magnitude of the Ca^{2+} response within the stimulated cell was unaltered between control ($160 \pm 28\%$, $n = 5$) and CPA ($147 \pm 19\%$, $n = 11$) treated groups ($P > 0.05$). Values are means \pm S.E.M from n animals. Analysis by Students' unpaired t-test; *, $P < 0.05$.

3.3.8 Effect of U-73122 on the spreading Ca^{2+} response induced by mechanical stimulation

Phospholipase C inhibition with U-73122 (10 μM), significantly attenuated the number of non-stimulated cells in which a rise in $[\text{Ca}^{2+}]_i$ was evoked (2.0 ± 1.4 cells, Figure 3.10a, $P < 0.05$, $n = 6$), while being without effect on the magnitude of the increase in fluo-4 fluorescence within the mechanically stimulated cell ($180 \pm 19\%$, Figure 3.10b, $P > 0.05$, $n = 6$).

3.3.9 Effect of U73122 on the spreading Ca^{2+} response in Ca^{2+} free conditions induced by mechanical stimulation

In the absence of extracellular Ca^{2+} , U-73122 (10 μM) also significantly attenuated the number of non-stimulated cells in which a rise in $[\text{Ca}^{2+}]_i$ was evoked (0.2 ± 0.4 cells, Figure 3.11a, $P < 0.05$, $n = 6$). As in control Ca^{2+} -free conditions, no rise in $[\text{Ca}^{2+}]_i$ was observed within the stimulated cell in the presence of U-73122 ($-14 \pm 6\%$, Figure 3.11b, $P > 0.05$, $n = 6$). In 6 out of 6 cases a decrease in fluorescence was observed.

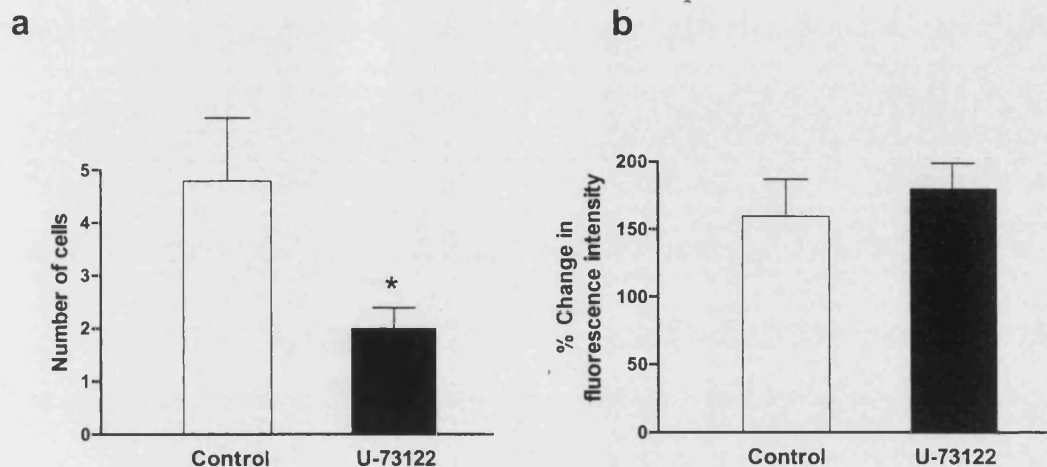


Figure 3.10 Effect of U-73122 on mechanical stimulation induced calcium responses in cultured rat aortic endothelial cells.

Effect of U-73122 (10 μ M) on a) the number of cells participating in the spreading Ca^{2+} response and b) the magnitude of the Ca^{2+} response within the stimulated cell. U-73122 restricted the stimulated rise in fluo-4 fluorescence to 2.0 ± 1.4 adjacent non-stimulated cells ($n = 6$). This differed significantly from control which spread to 4.8 ± 1.2 adjacent cells ($P < 0.05$, $n = 5$). The magnitude of the Ca^{2+} response within the stimulated cell was unaltered between control ($160 \pm 28\%$, $n = 5$) and U-73122 ($180 \pm 19\%$, $n = 6$) treated groups ($P > 0.05$). Values are means \pm S.E.M from n animals. Analysis by Students' unpaired t-test; *, $P < 0.05$.

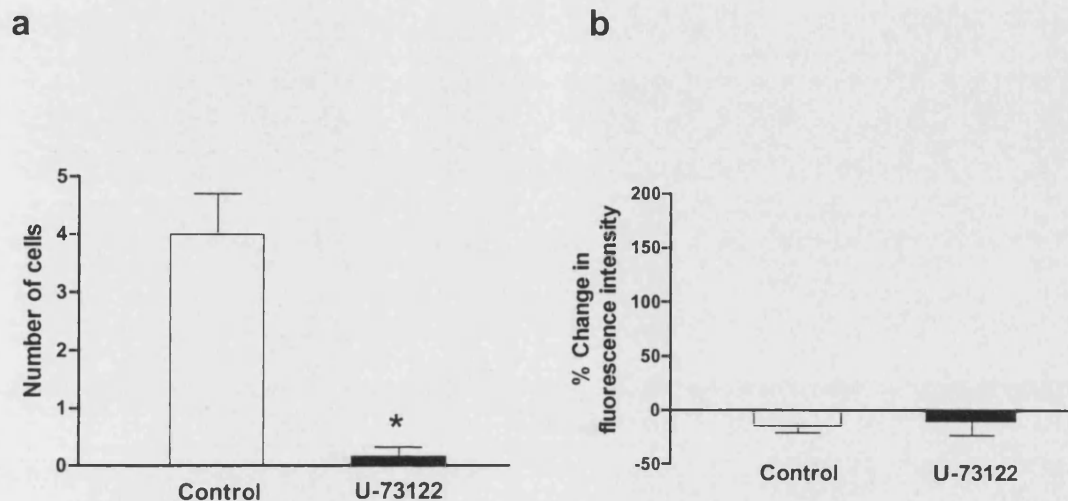


Figure 3.11 Effect of U-73122 in Ca^{2+} -free conditions on mechanical stimulation induced calcium responses in cultured rat aortic endothelial cells.

Effect of U-73122 (10 μM) on a) the number of cells participating in the spreading Ca^{2+} response and b) the magnitude of the Ca^{2+} response within the stimulated cell in Ca^{2+} -free conditions. U-73122 restricted the stimulated rise in fluo-4 fluorescence to 0.2 ± 0.4 adjacent non-stimulated cells ($n = 6$). This differed significantly from control which spread to 4.0 ± 0.7 adjacent cells ($P < 0.05$, $n = 5$). The inability of mechanical stimulation to evoke an increase in fluo-4 fluorescence within the stimulated cell in Ca^{2+} -free (control) conditions ($-10 \pm 13\%$, $n = 5$) was unaltered by the presence of U-73122 ($-14 \pm 6\%$, $n = 6$). Values are means \pm S.E.M from n animals. Analysis by Students' unpaired t-test; *, $P < 0.05$.

3.4 Discussion

Endothelial cell identification by uptake of DiI-Ac-LDL and staining with antibodies to vWF, demonstrated the enzymatic digestion and isolation procedure to yield an essentially pure population of endothelial cells with few contaminants. The ability of rat aortic endothelial cells in culture to permit the intercellular transfer of the fluorescent dye lucifer yellow, is a similar finding to that in porcine coronary artery endothelial cells (Domenighetti *et al.*, 1998) and indicates the presence of functional gap junction channels.

Application of a mechanical stimulus to a cell, is a convenient method by which to evoke an increase in $[Ca^{2+}]_i$ within a single stimulated cell (Boitano *et al.*, 1994; Demer *et al.*, 1993; Goligorsky, 1988; Hansen *et al.*, 1993; Isakson *et al.*, 2001). The finding that rat aortic endothelial cells responded to mechanical stimulation with a rise in $[Ca^{2+}]_i$, enabled the use of this method in the study of spreading Ca^{2+} responses between cells of this type. However, using this approach it is possible to wound rather than stimulate cells, leading to membrane rupture and the release of cytosolic constituents into the extracellular milieu (Isakson *et al.*, 2001; Sammak *et al.*, 1997). Several lines of evidence suggest that the stimulated cells of the present study were not mechanically wounded by this process, these include: the retention of fluo-4 within the cell following stimulation, recovery of cells to baseline $[Ca^{2+}]_i$ following stimulation and the ability of cells to respond to repeat stimuli. Mechanical wounding would also be expected to result in longer distance Ca^{2+} signalling than that observed in this study (Sammak *et al.*, 1997).

A mechanical force imposed upon an endothelial cell is also of physiological relevance, as endothelial cells are exposed to various physical forces *in vivo*, such as shear stress, changes in systolic and diastolic pressure during the cardiac cycle and deformational strain as the vessel wall distends and relaxes. The mechanosensor and the precise mechanism by which a mechanical stimulus evokes an increase in endothelial cell Ca^{2+} remains unclear, however several studies have implicated a role for the cytoskeleton in the detection of mechanical deformation, which may then be transduced to a membrane ion channel, protein kinase or phospholipase (Banes *et al.*, 1995; Diamond *et al.*, 1994). Stretch-activated Ca^{2+} channels may also play a role in the transduction of a mechanical stimulus to a rise in $[\text{Ca}^{2+}]_i$ within vascular endothelial cells (Lansman *et al.*, 1987).

The observed spread of the mechanically induced rise in $[\text{Ca}^{2+}]_i$ to adjacent non-stimulated cells, is in agreement with previous studies of intercellular Ca^{2+} signalling between cultured endothelial cells (Demer *et al.*, 1993; Domenighetti *et al.*, 1998; Moerenhout *et al.*, 2001). The apparent delay of 2.1 ± 0.5 s between the onset of the mechanically induced increase in $[\text{Ca}^{2+}]_i$ within the stimulated cell and the time of initiation of the rise in $[\text{Ca}^{2+}]_i$ within the adjacent cell, suggested that transfer of current between cells was unlikely to underlie the spreading Ca^{2+} response. The finding that the spreading responses were not uni-directional and not dependent upon the direction of the perfusion flow indicated that diffusion of an extracellular signal was also unlikely to underlie this response.

In support of the hypothesis of a gap junction mediated process, the presence of the gap junction uncoupling agent PA, inhibited the mechanically-evoked spreading Ca^{2+}

response. This effect was unlikely to be due to non-specific effects of the gap junction uncoupler upon Ca^{2+} mobilization within the cells, as increases in $[\text{Ca}^{2+}]_i$ in response to mechanical stimulation within the stimulated cell and the application of the Ca^{2+} -mobilizing agonist bradykinin, were unaffected by the presence of the fatty acid. The requirement for gap junctions in the transmission of intercellular Ca^{2+} signals between rat aortic endothelial cells is consistent with many published reports of intercellular Ca^{2+} signalling between endothelial cells from other species and vascular tissues (Demer *et al.*, 1993; Domenighetti *et al.*, 1998; Ying *et al.*, 1996).

In the absence of extracellular Ca^{2+} , mechanical stimulation was unable to evoke a rise in $[\text{Ca}^{2+}]_i$ within the stimulated cell, a finding also reported in calf pulmonary artery endothelial cells (Moerenhout *et al.*, 2001). This suggested extracellular Ca^{2+} influx, possibly *via* stimulation of stretch-activated ion channels (Lansman *et al.*, 1987), to underlie this response. However, the absence of extracellular Ca^{2+} was without effect on the spreading Ca^{2+} response, which still occurred in the absence of a rise in $[\text{Ca}^{2+}]_i$ within the stimulated cell.

Depletion of intracellular Ca^{2+} stores with the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) inhibitor CPA, suggested that intracellular Ca^{2+} release was required for the spread of the intercellular Ca^{2+} signal but not required to evoke a rise in $[\text{Ca}^{2+}]_i$ within the stimulated cell. These findings indicated that a calcium releasing factor, other than Ca^{2+} itself, was responsible for the generation of the intercellular Ca^{2+} signal. One such factor is IP_3 , a second messenger generated from the membrane phospholipid PIP_2 , following activation of membrane-bound phospholipase C (Berridge, 1993). The lack of effect of CPA on the mechanical stimulation-evoked rise in $[\text{Ca}^{2+}]_i$ within the stimulated

cell was in agreement with the finding that extracellular Ca^{2+} entry underlies this response. However, owing to the requirement for Ca^{2+} release from intracellular stores for the spread of increases in $[\text{Ca}^{2+}]_i$ to non-stimulated cells, the mechanical stimulus must have generated a calcium release factor.

Endothelial cells have been shown to respond to changes in cyclic stretch frequency (Brophy *et al.*, 1993) and mechanical perturbation (Diamond *et al.*, 1994) with PLC activation. The mechanism of activation is far from clear, but it has been suggested that distortion of the phospholipid bilayer may alter the normal configuration of the membrane-associated PLC or its access to phospholipid substrate (Demer *et al.*, 1993). The PLC inhibitor U-73122, was found to significantly reduce the number of non-stimulated cells for which an increase in $[\text{Ca}^{2+}]_i$ was evoked, without affecting the mechanically induced rise in $[\text{Ca}^{2+}]_i$ within the stimulated cell. These findings indicate that activation of PLC and the subsequent generation of IP_3 underlie the mechanical stimulation-evoked spreading Ca^{2+} response. So although mechanical stimulation activates PLC within the stimulated cell, PLC activation and intracellular Ca^{2+} release are not required to evoke an increase in $[\text{Ca}^{2+}]_i$ within this cell.

If indeed as indicated, IP_3 is generated in the mechanically stimulated cell, IP_3 -mediated Ca^{2+} release from intracellular stores would be expected to increase the $[\text{Ca}^{2+}]_i$ of that cell, even in the absence of extracellular Ca^{2+} . The observation that in Ca^{2+} -free conditions, mechanical stimulation was unable to evoke an increase in the $[\text{Ca}^{2+}]_i$ within stimulated cell is therefore most surprising, but is consistent with findings in similar studies (Moerenhout *et al.*, 2001; Sanderson *et al.*, 1990). Sanderson *et al.*, 1990 explained this observation on the basis of a reversal in the chemical gradient for Ca^{2+}

across the membrane, as a result of the absence of extracellular Ca^{2+} . The opening of mechanosensitive membrane channels in response to the application of a mechanical stimulus would therefore prevent the accumulation of intracellular Ca^{2+} in response to simultaneous PLC activation by allowing efflux of Ca^{2+} from the cell. In pulmonary artery endothelial cells, inhibition of mechanically evoked increases $[\text{Ca}^{2+}]_i$ by the removal of extracellular Ca^{2+} , and the subsequent reversal of this inhibition by the presence of Gd^{3+} and La^{3+} (Moerenhout *et al.*, 2001) is also in agreement with this hypothesis. The finding that an increase in $[\text{Ca}^{2+}]_i$ could not be initiated in the stimulated cell or adjacent cells in the presence of the PLC inhibitor U-73122 and Ca^{2+} -free conditions confirmed the requirement for extracellular Ca^{2+} and PLC activation in the initiation and spread of mechanically induced Ca^{2+} responses.

While a number of studies have implicated the involvement of PLC activation in endothelial cell Ca^{2+} signalling (Domenighetti *et al.*, 1998; Moerenhout *et al.*, 2001), differences between these findings and those of the present study arise upon consideration of the precise mechanism of the PLC mediated signal. Domenighetti *et al.* (1998) reported a regenerative process of Ca^{2+} release to underlie the intercellular transfer of changes in $[\text{Ca}^{2+}]_i$, implicating calcium-induced calcium release (CICR) and further activation of PLC by increases in $[\text{Ca}^{2+}]_i$. The short distance over which changes in $[\text{Ca}^{2+}]_i$ were communicated in the present study, suggests that the mechanism underlying this response is not regenerative, despite the ability of increases in $[\text{Ca}^{2+}]_i$ to directly activate PLC (Park *et al.*, 1992; Rhee & Choi, 1992). The spreading Ca^{2+} response was in fact restricted to a maximum of two cell widths from the stimulated cell and is therefore more likely to represent a process of simple diffusion by a long-range messenger such as IP_3 , the lifetime of which may be slowly reduced by IP_3 kinase

(Kasai & Petersen, 1994). Restrictions imposed by extensive intracellular buffering upon the long-range diffusion of Ca^{2+} , indicate the intercellular diffusion of this ion to be an unlikely signalling mechanism.

3.5 Conclusions

In summary this study has demonstrated that application of a mechanical stimulus to a single rat aortic endothelial cell initiates the spread of an intercellular Ca^{2+} signal through gap junction channels. In the stimulated cell, the rise in $[\text{Ca}^{2+}]_i$ is dependent upon extracellular Ca^{2+} entry. However, a rise in $[\text{Ca}^{2+}]_i$ within the stimulated cell is not required to evoke the spreading response which is dependent upon Ca^{2+} release from intracellular stores following the activation of PLC. These results indicate that diffusion of an intercellular messenger other than Ca^{2+} , possibly IP_3 , is the underlying mechanism responsible for the generation of this multicellular response (Figure 3.12).

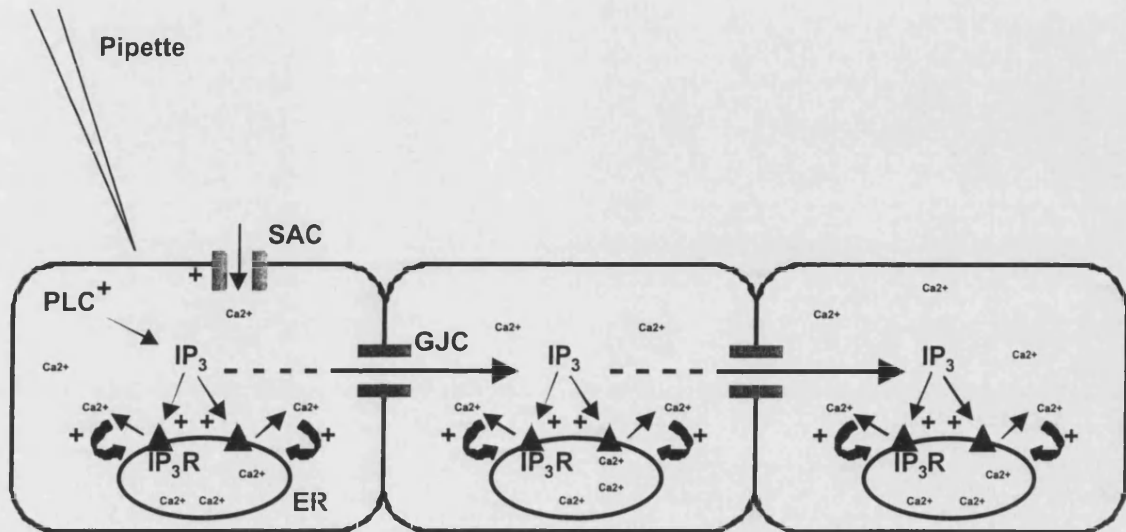


Figure 3.12 Schematic representation of the proposed mechanisms underlying spreading Ca^{2+} responses in rat aortic endothelial cells following mechanical stimulation.

Mechanical stimulation of a single endothelial cell evokes a rise in $[\text{Ca}^{2+}]_i$ within that cell in two ways; (i) by stimulating the opening of stretch activated channels (SAC) that permit Ca^{2+} influx from the extracellular space and (ii) by stimulating the generation of IP_3 from PLC, which releases Ca^{2+} from the endoplasmic reticulum (ER), *via* activation of IP_3 receptors (IP_3R). IP_3 diffuses to adjacent non-stimulated cells *via* gap junction channels (GJC), to evoke a rise in $[\text{Ca}^{2+}]_i$ within these cells. It is possible that the IP_3 stimulated increases in $[\text{Ca}^{2+}]_i$ are enhanced by a positive feedback effect of Ca^{2+} upon the IP_3Rs .

CHAPTER FOUR

**Contraction intensity influences the
mechanism for EDHF-mediated relaxation
in rat isolated small mesenteric arteries**

4.1 Introduction

In rat mesenteric arteries, endothelial cell hyperpolarization by agonists such as ACh reflects the opening of Ca^{2+} -activated K^+ channels (Hinton & Langton, 2003; White & Hiley, 2000) and can be amplified by the efflux of K^+ through inwardly rectifying K^+ channels (Crane *et al.*, 2003; Doughty *et al.*, 2001). The presence of myoendothelial gap junctions (Sandow & Hill, 2000; Sandow *et al.*, 2002) and the ability of hyperpolarizing current to be transferred between the endothelium and smooth muscle *via* these channels (Edwards *et al.*, 1999; Sandow *et al.*, 2002), suggests that electrical coupling between the two cell types may underlie EDHF-type responses in these arteries.

Alternatively, or perhaps in parallel, accumulation of extracellular K^+ resulting from the opening of endothelial Ca^{2+} -activated K^+ channels may evoke smooth muscle hyperpolarization and relaxation by stimulating the Na^+/K^+ -ATPase of these cells (Dora *et al.*, 2001) and thus act as an EDHF (Edwards *et al.*, 1998). However, as the level of background stimulation intensity with PE is increased, exogenous K^+ loses the ability to stimulate hyperpolarization and relaxation, but the EDHF response to ACh is not altered (Dora & Garland, 2001). Owing to the reversal of this effect by BK_{ca} blockade (Dora *et al.*, 2002; Richards *et al.*, 2001), it has been suggested that K^+ efflux from smooth muscle cells *via* BK_{ca} maximally activates the smooth muscle Na^+/K^+ -ATPase, thereby preventing further activation by K^+ released from the endothelium. In situations where the ability of exogenous K^+ to evoke relaxation is lost, persistent EDHF-mediated responses must reflect the existence of an additional pathway for smooth muscle hyperpolarization. The present study investigates the importance of gap junctions in these responses.

4.2 Methods

Third-order branches of rat superior mesenteric artery were isolated and mounted in a Mulvany-Halpern wire myograph (Danish Myotechnology, 400A) at a tension equivalent to 0.9 times the diameter of the vessel at 100 mmHg. Endothelium viability was assessed as >95% control relaxation to 1 μ M ACh in arteries contracted with PE (3 μ M). All experiments were performed in the presence of L-NAME (100 μ M). For detailed methodology see section 2.1.

4.3 Results

4.3.1 Effects of ACh and exogenous K⁺ at different levels of arterial contraction

In paired experiments, sub-maximal or low levels of PE-evoked contraction were fully reversed by cumulative additions of ACh (3 nM – 3 μ M, R_{\max} $97.2 \pm 0.7\%$, pD_2 7.2 ± 0.1 , $n = 10$, Figures 4.1a and 4.2a) or exogenous K⁺ (final bath concentration: 7.8 – 16.8 mM, R_{\max} $97.1 \pm 0.8\%$, $n = 10$, Figures 4.1b and 4.2b).

At high or near maximal levels of PE-evoked contraction, concentration-relaxation curves to ACh were shifted to the right, while maximum relaxation was maintained (R_{\max} $95.1 \pm 0.5\%$, pD_2 7.0 ± 0.1 , $n = 11$, Figures 4.1c and 4.2a). In contrast, relaxation to exogenous K⁺ was abolished at high tension (R_{\max} $7.2 \pm 2.1\%$, $n = 11$, Figures 4.1d and 4.2b).

The concentrations of PE used and the tension evoked (mN) at high and low levels of PE-evoked contraction are given in Tables 4.1 and 4.2.

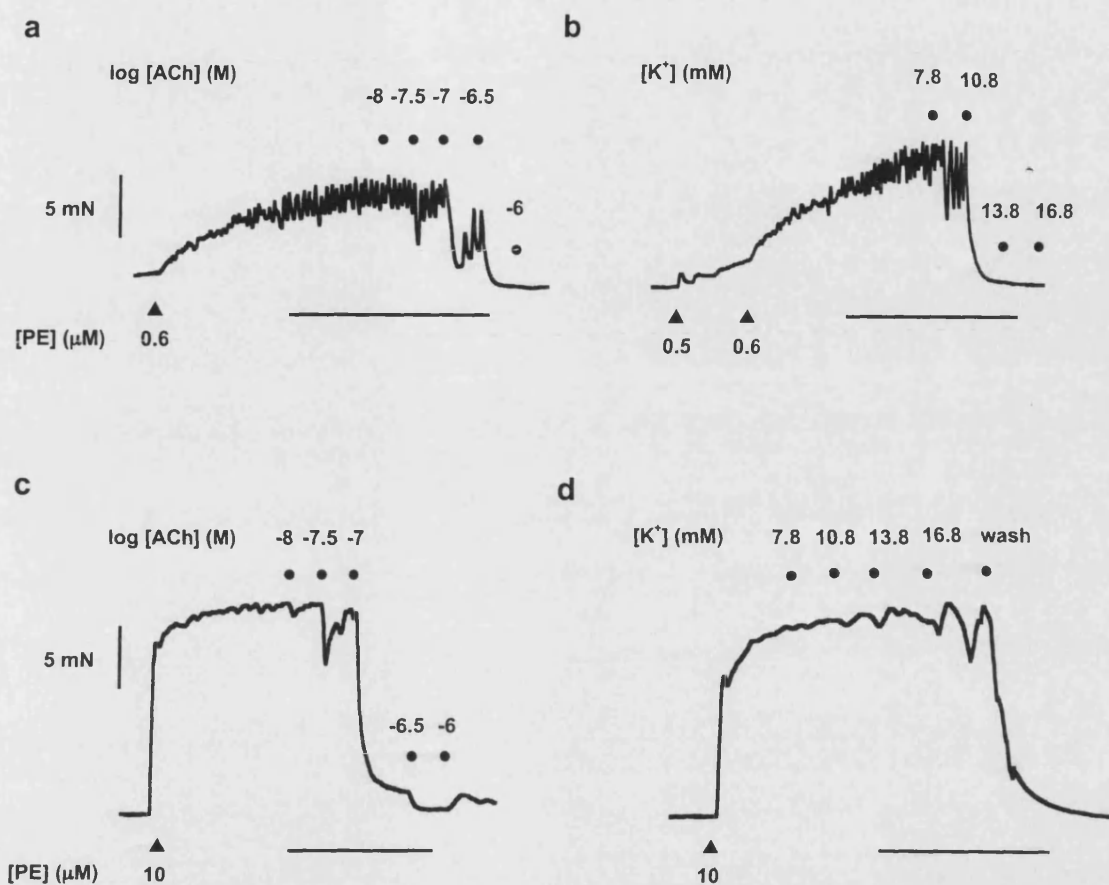


Figure 4.1 Representative traces showing relaxation evoked by ACh and K^+ at low and high levels of contraction stimulated by PE.

At both low and high levels of contraction, the cumulative application of increasing concentrations of ACh fully relaxed rat mesenteric arteries (a and c respectively). Relaxation evoked by the cumulative addition of K^+ at low tension was abolished when arteries were maximally contracted (b and d respectively). The Krebs contained 4.8 mM K^+ . Experiments were performed in the presence of L-NAME (100 μ M). Bars = 5 min.

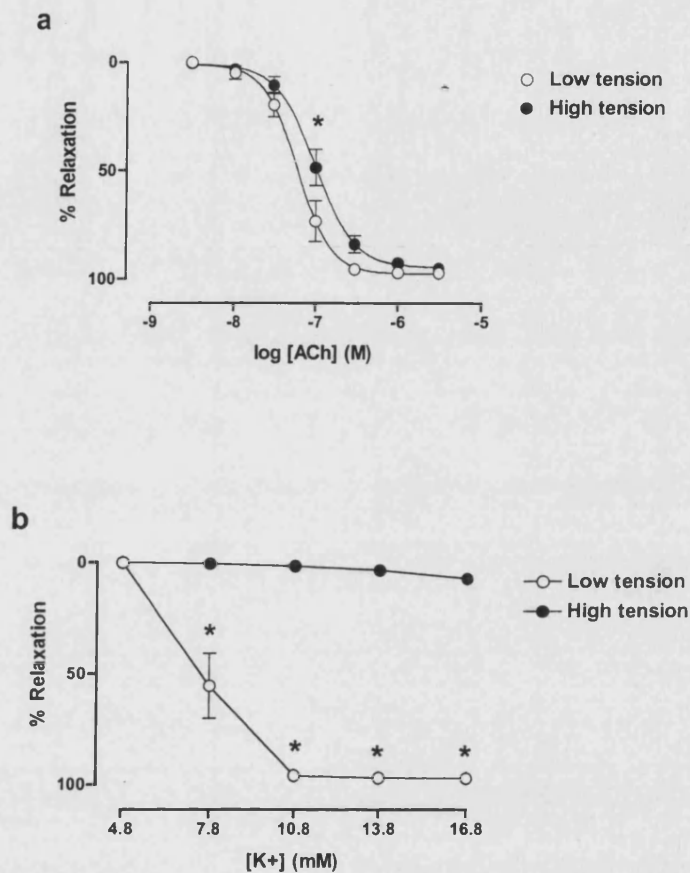


Figure 4.2 Effect of contraction intensity on relaxation evoked by ACh and K⁺.

Cumulative concentration-response curves to ACh and K⁺ in rat mesenteric arteries contracted with PE. Values for ACh and K⁺ were pooled from Figures 4.5 and 4.6 for low ($n = 10$) and high ($n = 11$) tension responses. The ACh concentration response curve (a) was shifted to the right at high tension, while relaxation to K⁺ (b) was almost fully abolished. The Krebs solution contained 4.8 mM K⁺ and L-NAME (100 μ M). Values are means \pm S.E.M from n arteries. Analysis by Students' unpaired t-test to compare pD_2 values, and one-way ANOVA with Bonferroni's post-test to compare individual points between curves. Significant differences between points indicated by *, $P < 0.05$ versus low tension.

Table 4.1 Effect of CBX on contraction mediated by PE.

	<i>Low Contraction</i>			<i>High Contraction</i>		
	Tension (mN)	[PE] (μ M)	<i>n</i>	Tension (mN)	[PE] (μ M)	<i>n</i>
<i>ACh</i>						
L-NAME	10.8 \pm 0.6	0.4 \pm 0.1	5	19.0 \pm 2.5	10.0 \pm 0.0	6
+ CBX	8.9 \pm 0.7	0.6 \pm 0.1	5	18.6 \pm 1.8	10.0 \pm 0.0	6
<i>K⁺</i>						
L-NAME	8.9 \pm 0.5	0.4 \pm 0.1	5	18.8 \pm 2.3	10.0 \pm 0.0	6
+ CBX	9.3 \pm 0.9	0.6 \pm 0.1	5	17.1 \pm 2.3	10.0 \pm 0.0	6

Average levels of low and high tension, and concentrations of PE used, before measuring relaxation to either ACh or K⁺. Responses from an artery were obtained both before (L-NAME, 100 μ M) and after exposure for 30 minutes to CBX (100 μ M). Values are means \pm S.E.M from *n* arteries.

Table 4.2 Effect of Gap 27 on contraction mediated by PE.

	<i>Low Contraction</i>			<i>High Contraction</i>		
	Tension (mN)	[PE] (μ M)	<i>n</i>	Tension (mN)	[PE] (μ M)	<i>n</i>
<i>ACh</i>						
L-NAME	10.8 \pm 1.2	0.3 \pm 0.0	5	15.2 \pm 1.1	10.0 \pm 0.0	5
+ Gap 27	10.3 \pm 1.4	0.3 \pm 0.0	5	14.4 \pm 1.2	10.0 \pm 0.0	5
<i>K⁺</i>						
L-NAME	9.1 \pm 0.8	0.3 \pm 0.1	5	15.4 \pm 1.2	10.0 \pm 0.0	5
+ Gap 27	10.0 \pm 1.3	0.3 \pm 0.0	5	15.4 \pm 1.3	10.0 \pm 0.0	5

Average levels of low and high tension, and concentrations of PE used, before measuring relaxation to either ACh or K⁺. Responses from an artery were obtained both before (L-NAME, 100 μ M) and after exposure for 2 hours to Gap 27 (300 μ M). Values are means \pm S.E.M from *n* arteries.

4.3.2 Effects of gap junction uncoupling agents: PA, CBX and Gap 27 on arterial contraction.

The presence of PA (50 μ M) induced a significant rightward shift in the concentration-response curve to PE (10 nM – 10 μ M, pD_2 values for control and PA treated arteries were 6.0 ± 0.0 and 5.7 ± 0.1 respectively, $P < 0.05$, $n = 3$, Figure 4.3a). Contraction to phenylephrine was however unaffected by exposure to CBX (100 μ M, pD_2 values for control and CBX treated arteries were 6.0 ± 0.1 and 5.9 ± 0.1 respectively, $P > 0.05$, $n = 6$, Figure 4.3b) or Gap 27 (300 μ M, pD_2 values for control and Gap 27 treated arteries were 5.8 ± 0.1 and 5.7 ± 0.1 respectively, $P > 0.05$, $n = 3$, Figure 4.3c).

Due to the attenuating effect of PA on PE-evoked contraction, the gap junction uncoupling agent was considered unsuitable for use in the functional studies of this chapter.

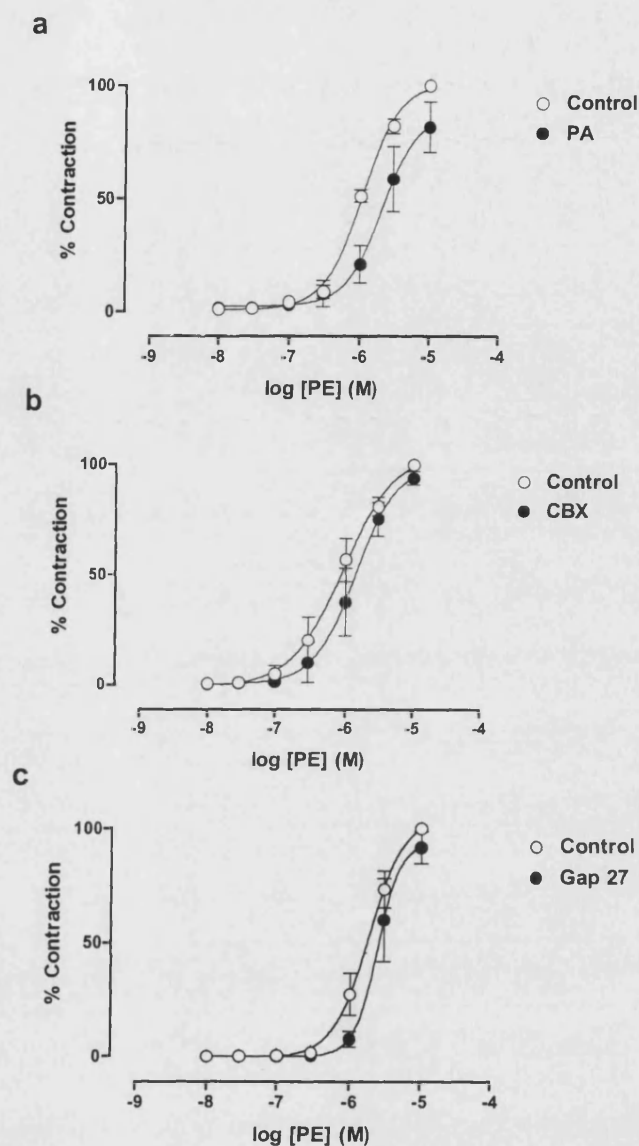


Figure 4.3 Effect of the gap junction uncoupling agents: PA, CBX and Gap 27 on contraction evoked by PE.

PE concentration-response curves were shifted to the right in the presence of PA (a, 50 μM , $n = 3$), but were unaffected by the presence of CBX (b, 100 μM , $n = 6$) or Gap 27 (c, 300 μM , $n = 3$). All experiments were performed in the presence of L-NAME (100 μM). Values are means \pm S.E.M from n arteries. Analysis by Students' paired t-test to compare pD_2 values, and one-way ANOVA with Bonferroni's post-test to compare individual points between curves. Significant differences between points indicated by *, $P < 0.05$.

4.3.3 Effect of CBX on relaxation evoked by ACh and exogenous K⁺

In the additional presence of the gap junction uncoupler CBX (100 μ M), relaxation to ACh was unaffected, except at 100 nM, in arteries contracted to low levels (R_{\max} 93.0 ± 3.2 , pD_2 6.9 ± 1.4 , $n = 5$, Figures 4.4a and 4.5a), while during high levels of contraction to PE, relaxation to ACh was abolished (R_{\max} 10.3 ± 7.7 , $n = 6$, Figures 4.4c and 4.5b). CBX did not affect the ability of K⁺ to evoke relaxation during low levels of contraction to PE (Figure 4.5c). During high levels of contraction, the apparent relaxation to K⁺ at 13.8 and 16.8 mM was not significantly different from control (Figure 4.5d).

During high levels of contraction to PE, when CBX (with L-NAME present) abolished relaxation to ACh, full relaxation could be evoked with the K_{ATP} channel opener levcromakalim (1 μ M, $97.1 \pm 1.0\%$, $n = 6$), suggesting that the ability of smooth muscle cell hyperpolarization to cause relaxation was not affected. The maximum contraction achieved in all experiments in which arteries were exposed to CBX was 18.7 ± 1.6 mN ($n = 11$).

In the presence of CBX, the concentrations of PE used and the tension evoked (mN) at high and low levels of PE-evoked contraction are given in Table 4.1.

4.3.4 Effect of Gap 27 on relaxation evoked by ACh and exogenous K⁺

Relaxation to ACh was unaffected by the presence of the connexin mimetic peptide Gap 27 (300 μ M) during low levels of arterial contraction (Figures 4.4b and 4.6a), while at high levels of contraction the concentration-relaxation curve for ACh was significantly shifted to the right without a change in maximal relaxation (R_{\max} $79.7 \pm 15.1\%$, pD_2 6.5 ± 0.1 , $n = 5$, Figures 4.4d & 4.6b). As with CBX, relaxation to exogenous K⁺ at low and high levels of PE-mediated contraction, was unaffected by the presence of Gap 27 (Figure 4.6c and 4.6d). The maximum contraction achieved in all experiments in which arteries were exposed to Gap 27 was 17.8 ± 1.0 mN ($n = 10$).

In the presence of Gap 27, the concentrations of PE used and the tension evoked (mN) at high and low levels of PE-evoked contraction are given in Table 4.2.

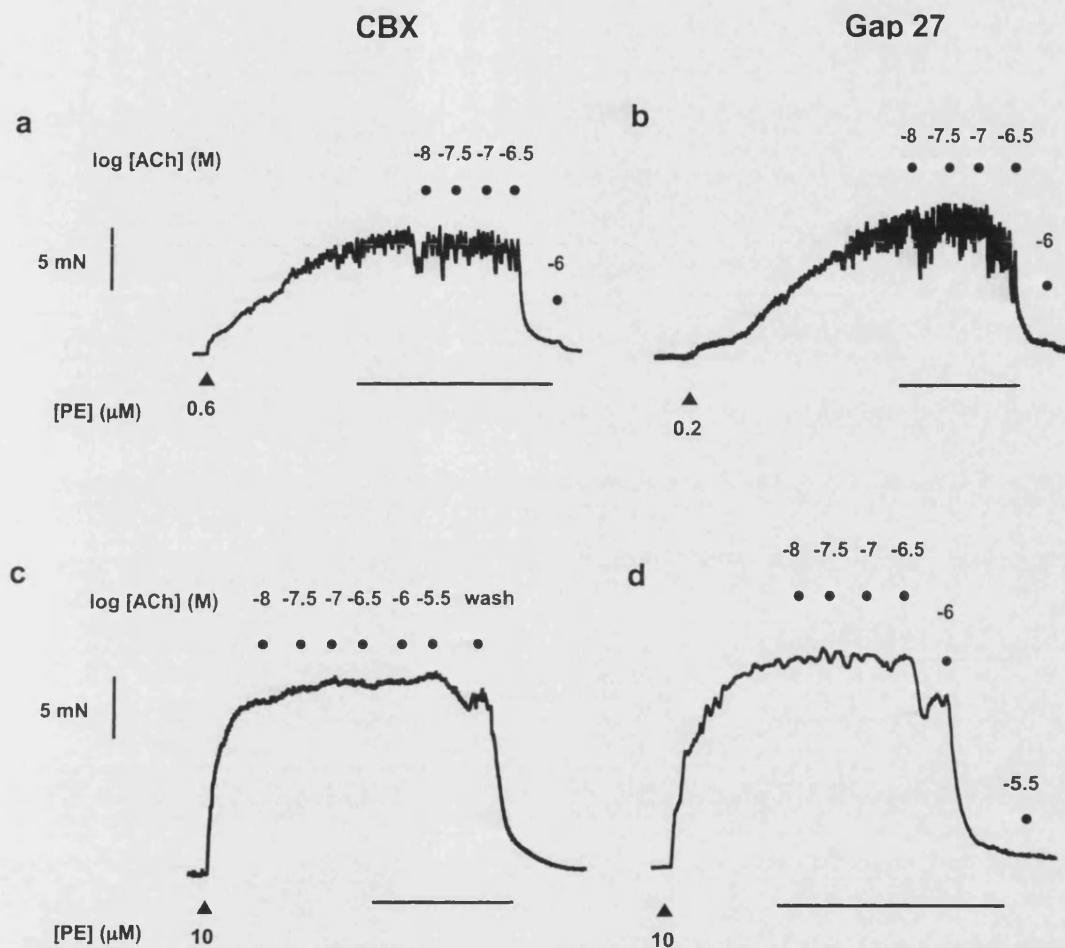


Figure 4.4 Representative traces showing the effect of CBX and Gap 27 on EDHF-mediated relaxation to ACh at low and high levels of contraction stimulated by PE.

In the presence of CBX (100 μ M), ACh-evoked relaxation persisted at low tension, while at high tension, was completely abolished (a and c respectively). Gap 27 (300 μ M) was also without effect on ACh-evoked relaxation at low tension (b). At high tension the peptide significantly attenuated relaxation to ACh, but was without effect on the maximal response (d). Experiments were performed in the presence of L-NAME (100 μ M). Bars = 5 min.

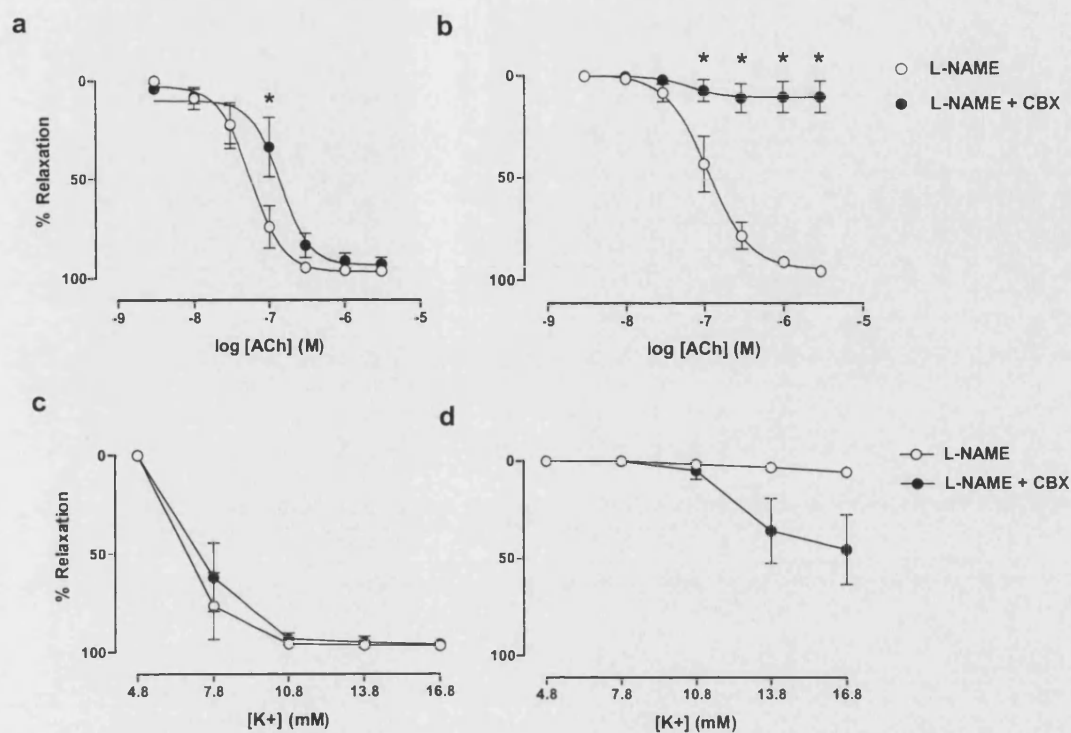


Figure 4.5 Effect of CBX on relaxation evoked by ACh and K⁺.

Cumulative concentration response curves to ACh and K⁺ in rat mesenteric arteries contracted with PE. The average concentrations of PE used to stimulate low and high levels of contraction are summarized in Table 4.1. After stimulation of low levels of contraction, addition of CBX (100 μ M) was without effect on the concentration-relaxation curve to ACh, except at 100 nM (a, *n* = 5). At high levels of contraction, relaxation to ACh was abolished by CBX (b, *n* = 6). The relaxation to cumulative additions of K⁺ was not affected by CBX at either low (c) or high (d) levels of contraction (*n* = 5 & 6 respectively). The Krebs solution contained 4.8 mM K⁺. All experiments were performed in the presence of L-NAME (100 μ M). Values are means \pm S.E.M from *n* arteries. Analysis by Students' paired *t*-test to compare *p*D₂ values, and one-way ANOVA with Bonferroni's post-test to compare individual points between curves. Significant differences between points indicated by *, *P* < 0.05 versus L-NAME.

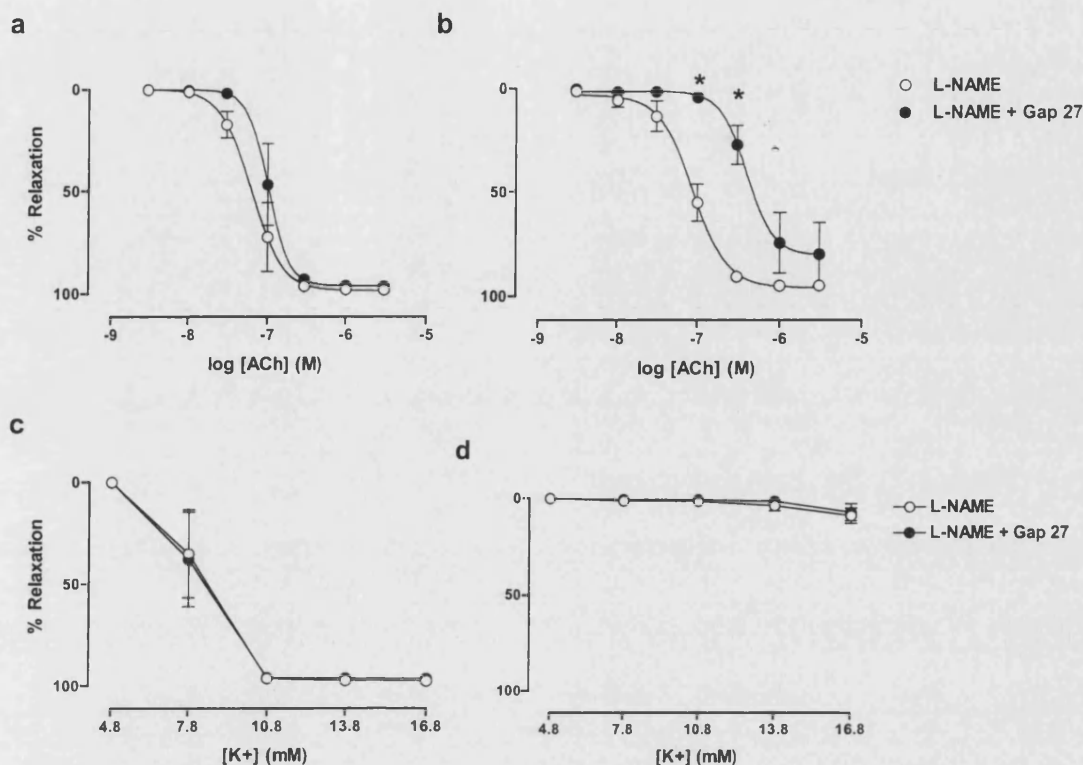


Figure 4.6 Effect of Gap 27 on relaxation evoked by ACh and K⁺.

Cumulative concentration response curves to ACh and K⁺ in rat mesenteric arteries contracted with PE. The average concentrations of PE used to stimulate low and high levels of contraction are summarized in Table 4.2. After stimulation of low levels of contraction, addition of Gap 27 (300 μ M) had no effect on the concentration-relaxation curve to ACh (a, $n = 5$). At high levels of contraction, relaxation to ACh was shifted to the right (b, $P < 0.01$, $n = 5$). Relaxation to cumulative additions of K⁺ was not affected by Gap 27 at either low (c) or high (d) levels of contraction ($n = 5$). The Krebs solution contained 4.8 mM K⁺. All experiments were performed in the presence of L-NAME (100 μ M). Values are means \pm S.E.M from n arteries. Analysis by Students' paired t-test to compare pD₂ values, and one-way ANOVA with Bonferroni's post-test to compare individual points between curves. Significant differences between points indicated by *, $P < 0.05$ versus L-NAME.

4.4 Discussion

These data are consistent with the suggestion that in rat mesenteric small arteries, the contribution of K^+ to EDHF-mediated relaxation diminishes as arterial contraction increases. The persistent ability of ACh to evoke EDHF relaxation can be explained by the presence of another pathway that appears to operate at all levels of contraction, but can be most clearly identified as contraction intensity increases. The sensitivity of this K^+ -independent relaxation to CBX and Gap 27 indicates a functional role for the myoendothelial gap junctions present in this artery.

The identity of EDHF in rat mesenteric arteries has been the subject of considerable debate. One consistent finding is that in the presence of the NO synthase inhibitor L-NAME, endothelium-dependent relaxation to ACh is abolished by the combined presence of apamin and charybdotoxin (Doughty *et al.*, 1999; Waldron & Garland, 1994) and more recently by apamin and the selective blocker of intermediate conductance Ca^{2+} -activated K^+ channels, TRAM-39 (Hinton & Langton, 2003). Consistent with this finding is the expression profile for the various Ca^{2+} -activated K^+ channels (K_{Ca}) in this artery. It appears that SK_{Ca} and IK_{Ca} are confined to the endothelium (Dora & Garland, 1999; Doughty *et al.*, 1999; Hinton & Langton, 2003; Walker *et al.*, 2001) whereas BK_{Ca} are found only in the smooth muscle layers (Walker *et al.*, 2001). Thus, since endothelial cell hyperpolarization is a crucial early step in the EDHF pathway (Edwards *et al.*, 1998; Hinton & Langton, 2003; White & Hiley, 2000), the efflux of K^+ from this layer has been suggested to play an important role in the EDHF pathway (Burnham *et al.*, 2002; Dora *et al.*, 2002; Edwards *et al.*, 1998). Indeed, in rat mesenteric arteries contracted with PE, raising the extracellular K^+ concentration

by up to 12 mM evokes smooth muscle hyperpolarization and relaxation by activating an ouabain-sensitive Na^+/K^+ ATPase and a Ba^{2+} -sensitive inwardly rectifying K^+ current (K_{IR}), mimicking an EDHF response (Dora & Garland, 2001; Edwards *et al.*, 1998). In this artery, functional K_{IR} activity appears to be restricted to the endothelium, with direct smooth muscle hyperpolarization to K^+ explained by the stimulation of the Na^+/K^+ -ATPase (Crane *et al.*, 2003; Dora & Garland, 2001). However, once depolarization and contraction to phenylephrine exceeds around -40 mV and 10 mN respectively, the repolarizing and relaxing effects of K^+ are blocked (Dora & Garland, 2001). This effect has been attributed to the recruitment of smooth muscle K_{Ca} channels as a result of increasing PE stimulation. The resulting local elevation in $[\text{K}^+]_o$ can activate the Na^+/K^+ -ATPase such that any additional stimulation of the Na^+/K^+ -ATPase by K^+ is blocked (Dora *et al.*, 2002; Weston *et al.*, 2002). The present study indicates that under such conditions, which may well occur physiologically, the functional importance of the K^+ -independent component of the EDHF response assumes predominance.

The most likely identity of a parallel pathway for EDHF-mediated relaxation is suggested by morphological evidence in rat mesenteric arteries for both homocellular and heterocellular gap junctions (Sandow *et al.*, 2002). These gap junctions can potentially allow the spread of hyperpolarizing current between the cells of the artery wall, including between the endothelium and the adjacent smooth muscle cells. Indeed, gap junction blockade with glycyrrhetic acid derivatives or connexin mimetic peptides does suggest an inhibition of the transfer of hyperpolarizing current between the two cell types (Dora *et al.*, 1999; Edwards *et al.*, 1999; Sandow *et al.*, 2002; Yamamoto *et al.*, 1999). This pathway may therefore permit smooth muscle cell hyperpolarization and relaxation in conditions where K^+ cannot. In this study, the gap junction uncoupler

CBX (a water-soluble derivative of 18β -GA), and the connexin mimetic peptide Gap 27, were used to assess the potential functional role of myoendothelial gap junctions in EDHF-mediated relaxation, although an additional action of these agents at homocellular gap junctions (between smooth muscle cells and between endothelial cells) cannot be excluded. The finding that neither CBX nor Gap 27 had an effect on contraction to PE, suggested that gap junctions between smooth muscle cells are not essential for the development of contraction, or are not uncoupled by these agents in rat mesenteric arteries.

The finding that at lower levels of PE contraction, both CBX and Gap 27 failed to inhibit relaxation to ACh or exogenously applied K^+ , suggests that the inhibitory effects of these uncouplers on relaxation to ACh at high contraction, are not simply a non-specific action on endothelial or smooth muscle cell Ca^{2+} handling. This finding is also supported by the ability of hyperpolarization to drive relaxation when the K_{ATP} channel opener levcromakalim was applied in the presence of CBX at near maximal levels of contraction. The inhibitory effects of CBX and Gap 27 upon ACh evoked relaxation only occurred when arteries were near to maximal contraction and unable to relax to the application of exogenous K^+ . However, the inhibitory effect of CBX was complete abolition of ACh induced relaxation, while that of Gap 27 was to induce a rightward shift of the concentration-response curve to ACh without depressing the maximal relaxation. These differences might be explained by the ability of CBX, which is a non-selective gap junction uncoupler, to potentially block all the myoendothelial gap junctions (in addition to homocellular gap junctions), while Gap 27 will only inhibit gap junction channels composed from connexins 37 and 43. The presence of Gap 27 may therefore still permit EDHF induced smooth muscle cell hyperpolarization and

relaxation via gap junctions composed either entirely or partly from other connexin proteins.

The ability of 10.8 mM K⁺ to evoke full relaxation in arteries contracted to low levels of contraction is consistent with the findings of Dora and Garland (2001), and further illustrates the possibility that K⁺ may be an EDHF in this artery. Another consistent finding is that relaxation to 10.8 mM K⁺ was blocked at high levels of contraction with PE. However, in contrast to the above study, relaxation to further elevations in [K⁺]_o were also blocked by high levels of contraction. This may be explained by the higher concentration of PE (10 μM) used in the present study to evoke near maximal contraction. Interestingly, using the same artery, other groups have reported an inability of K⁺ to stimulate relaxation against 10 μM PE (Doughty *et al.*, 2000; Lacy *et al.*, 2000). The inability of increasing concentrations of K⁺ to overcome the blockade, suggests that in this artery ACh-induced EDHF-mediated relaxation cannot be mimicked by K⁺ and can therefore only be partly explained by this ion acting as an EDHF after efflux from the endothelium.

4.5 Conclusions

In summary, these results in rat mesenteric arteries indicate that the intensity of smooth muscle contraction can have a significant influence on the mechanism responsible for EDHF-mediated relaxation. As the ability of K^+ to evoke relaxation diminishes with arterial contraction, the ability of gap junction uncoupling agents to inhibit EDHF-mediated relaxation is increased. These data are consistent with an ability of K^+ and gap junctions to operate in parallel to evoke EDHF-mediated smooth muscle hyperpolarization and relaxation and may help to explain inconsistencies between studies attempting to characterise the EDHF response in the rat mesenteric artery.

CHAPTER FIVE

**ATP release from smooth muscle underlies
vasoconstriction-evoked NO release in rat
mesenteric arteries**

5.1 Introduction

Increases in endothelial cell $[Ca^{2+}]_i$ stimulate the release of vasodilators such as NO and EDHF (Feletou & Vanhoutte, 1999; Fleming & Busse, 1999). In this way, activation of endothelial receptors associated with Ca^{2+} mobilization, or in some cases, flow-mediated increases in shear stress, result in the release of these factors and arterial relaxation (Geiger *et al.*, 1992; James *et al.*, 1995). In addition to direct activation of endothelial cells, it is also clear that elevations in smooth muscle cell Ca^{2+} and vasoconstriction resulting from NA and PE stimulation, can also cause an increase in endothelial cell $[Ca^{2+}]_i$ and generation of NO (Boer *et al.*, 1999; Dora *et al.*, 1997; Dora *et al.*, 2000; Schuster *et al.*, 2001; Tuttle & Falcone, 2001). Such increases in endothelial cell $[Ca^{2+}]_i$ have been suggested to result from the diffusion of Ca^{2+} or a Ca^{2+} release signal from the smooth muscle via myoendothelial gap junctions (Dora *et al.*, 1997; Schuster *et al.*, 2001), release from the smooth muscle of a factor which can induce a rise in endothelial cell $[Ca^{2+}]_i$ (Boer *et al.*, 1999), or the direct stimulation of α_1 - or α_2 -adrenoceptors on the endothelium (Cocks & Angus, 1983; Tuttle & Falcone, 2001). Indeed there is much evidence demonstrating the modulation of agonist stimulated smooth muscle contraction by the concomitant release of endothelium-derived relaxant factors (EDRFs, Angus *et al.*, 1986; Boer *et al.*, 1999; Dora *et al.*, 2000; Tuttle & Falcone, 2001). These studies have demonstrated this by evoking greater constriction to PE or NA following NOS inhibition or endothelial removal. However, in addition to stimulated release, basal release of NO can be responsible for the depression of vasoconstrictor responses (for review see Martin, 1988). In rat mesenteric arteries, the 2 - 3 fold increase in smooth muscle cGMP formation seen following the addition of PE, suggests that in these arteries NO release can be evoked by a vasoconstrictor

stimulus (Plane *et al.*, 1996). Schuster *et al.* (2001) implicated a role for gap junctions in this response, but did not directly examine the effect of gap junction blockade on the production of EDRFs. Therefore while there is no direct evidence to suggest that these channels are involved in the release of EDRFs during arterial constriction, the possibility cannot be refuted. Equally, endothelial activation by a diffusible smooth muscle derived factor cannot be excluded.

Within the artery wall, the close apposition of smooth muscle and endothelial cells raises the possibility that the release of ATP from either population of cells may act as an autocrine or paracrine signalling factor between and within both cell types (Schwiebert, 2001). Calcium-mobilising agonists such as angiotensin II, NA and ATP itself have been demonstrated to evoke ATP release from vascular endothelial cells, taenia coli smooth muscle cells and ileal longitudinal muscle segments (Bodin & Burnstock, 1996; Katsuragi *et al.*, 1991; Matsuo *et al.*, 1997; Shinozuka *et al.*, 1997), suggesting that calcium mobilization may be a general stimulus for ATP release (Katsuragi *et al.*, 2002). In a similar manner, vasoconstrictor agents that raise intracellular calcium, may evoke ATP release from vascular smooth muscle cells and stimulate the production of EDRFs *via* endothelial purinergic receptors (Buvinic *et al.*, 2002; Malmsjo *et al.*, 2002; Marrelli, 2001; Stanford *et al.*, 2001). The present study addresses the involvement of gap junctions versus an extracellular signalling mechanism in vasoconstriction-evoked NO release in rat mesenteric arteries.

5.2 Methods

Third-order branches of rat superior mesenteric artery were isolated and mounted in a Mulvany-Halpern wire myograph (Danish Myotechnology, 400A) at a tension equivalent to 0.9 times the diameter of the vessel at 100 mmHg. Endothelium viability was assessed as >95% control relaxation to 1 μ M ACh in arteries contracted with PE (3 μ M). For detailed methodology on isometric tension recordings see section 2.1. Mesenteric smooth muscle and endothelial cells were isolated and labelled with the fluorescent markers quinacrine and mitofluor as described in sections 2.3.1, 2.4.1 and 2.4.3. Calcium imaging experiments on isolated cells are detailed in section 2.5. ATP release from rat mesenteric arteries was detected by luciferase driven bioluminescence according to the methodology described in section 2.2.

5.3 Results

5.3.1 Effect of inhibition of NO synthesis and soluble guanylate cyclase on phenylephrine or K⁺ induced contraction.

Addition of L-NAME (100 μ M) to arteries pre-contracted to $\leq 25\%$ of maximum contraction with PE (300 nM-3 μ M) or K⁺ (25-45 mM) induced further contraction that could not be evoked in non-contracted arteries at normalized tension (Table 5.1 and Figure 5.1). The level of pre-contraction evoked by either PE or K⁺ was not positively correlated with the subsequent contraction evoked by L-NAME as indicated by R² values closer to 0 than 1 (R² = 0.29, n = 8 and R² = 0.18, n = 6 respectively). Inhibition of soluble guanylate cyclase with ODQ (10 μ M) also induced contraction of similar magnitude to L-NAME in PE and K⁺ stimulated arteries (Table 5.1). Concentration response curves to PE (10 nM – 10 μ M) and K⁺ (25 mM – 55 mM) were similarly augmented following incubation with either L-NAME or ODQ (Figure 5.2). Endothelium removal abolished contractions evoked by L-NAME in PE pre-stimulated arteries (P < 0.05, n = 5), while contraction to the NOS inhibitor persisted in the absence of endothelium following K⁺ stimulation (P > 0.05, n = 5, Table 5.1). The nNOS inhibitor propyl-L-arginine (PLA, 1 μ M) evoked contractions of similar magnitude to L-NAME in arteries pre-stimulated with K⁺, but failed to evoke a response in PE contracted arteries (Table 5.1). L-NAME evoked contractions were unaffected by the presence of the nNOS inhibitor in both PE and K⁺ pre-contracted arteries (Table 5.1).

Under isobaric conditions, mesenteric arteries pre-stimulated with PE also contracted in response to L-NAME (100 μ M, $23.3 \pm 7.5\%$ of maximum contraction, $n = 2$, personal communication with Simon Mather).

5.3.2 Calcium responses to agonist stimulation in isolated endothelial and smooth muscle cells

Fluo-4 (50 μ M) loaded rat mesenteric smooth muscle cells responded with a $28.6 \pm 4.2\%$ ($n = 3$) and $34.2 \pm 8.8\%$ ($n = 3$) increase in fluorescence intensity to stimulation with PE (10 μ M) and K^+ (40 mM) respectively, but failed to respond to ACh (10 μ M, $1.3 \pm 0.7\%$, $n = 1$). In contrast, endothelial cells responded with an increase in fluo-4 intensity to stimulation with ACh ($47.3 \pm 13.7\%$, $n = 3$), while PE ($0.7 \pm 0.4\%$, $n = 3$) and K^+ ($2.3 \pm 0.6\%$, $n = 3$) were without effect. Stimulation with ATP (10 μ M) evoked a rise in intracellular calcium in both endothelial and smooth muscle cells ($34.5 \pm 1.3\%$, $n = 3$ and $30.7 \pm 3.3\%$, $n = 3$ respectively, Figure 5.3).

Table 5.1 Contraction evoked during blockade of NO synthesis or soluble guanylate cyclase in rat mesenteric arteries pre-contracted with PE or K⁺.

	PE	K ⁺
<i>L-NAME (control)</i>	53.8 ± 4.7 (n = 8)	36.1 ± 9.2 (n = 6)
<i>L-NAME (denuded)</i>	4.5 ± 3.2 (n = 5)*	36.7 ± 3.0 (n = 5)
<i>ODQ</i>	56 ± 2.2 (n = 3)	42.7 ± 7.8 (n = 3)
<i>PLA</i>	0.0 ± 0.0 (n = 3)*	36.0 ± 6.3 (n = 3)
<i>L-NAME (+PLA)</i>	36.3 ± 0.7 (n = 3)	45.5 ± 6.8 (n = 3)

Arteries were initially contracted to ≤ 25% of maximum contraction with either phenylephrine (PE, 300 nM – 3 μM) or K⁺ (25 – 45 mM) and were allowed to plateau before the addition of L-NAME (100 μM), ODQ (10 μM) or the nNOS selective inhibitor, propyl-L-arginine (PLA, 1 μM). Values are expressed as percentage of maximum contraction and are means ± S.E.M from *n* arteries. Analysis by one-way ANOVA with Bonferroni's post test. Significant differences against L-NAME control indicated by * (*P* < 0.05).

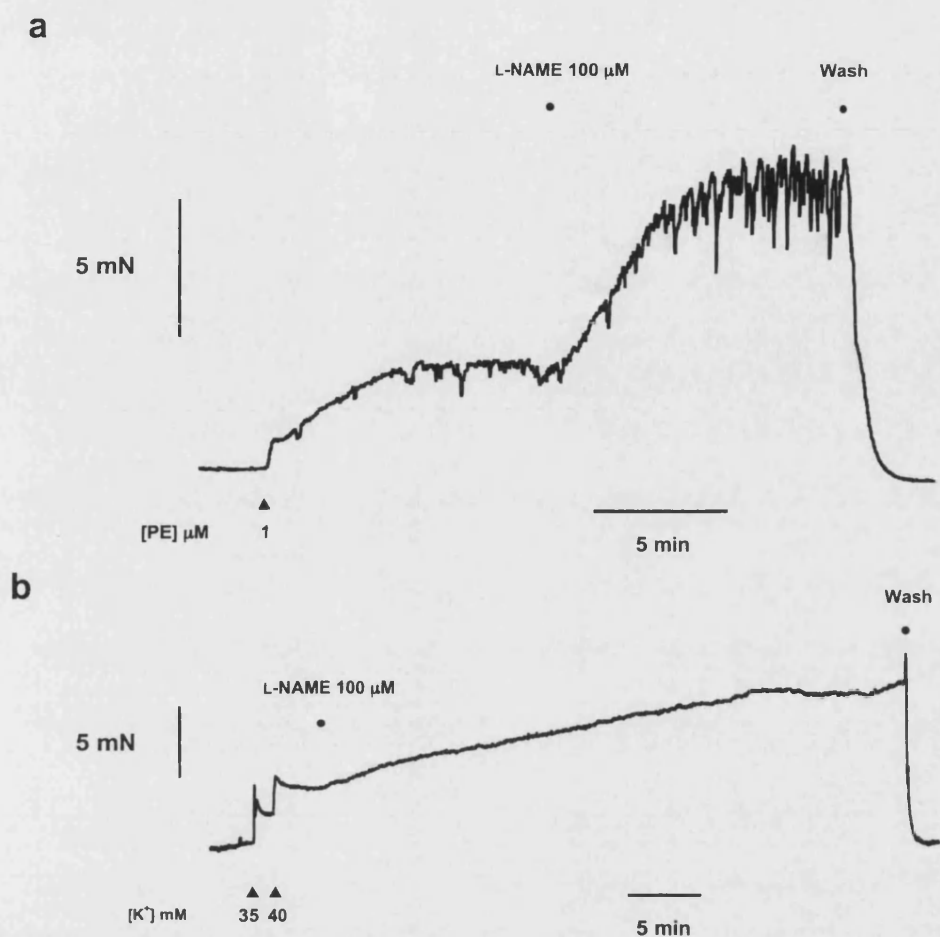


Figure 5.1 Representative traces showing the effect of addition of L-NAME to rat mesenteric arteries pre-contracted with PE or K^+ .

Addition of L-NAME (100 μ M) induced further contraction in arteries pre-contracted with phenylephrine (PE) or K^+ . In the presence of K^+ , L-NAME induced contraction was slower to develop.

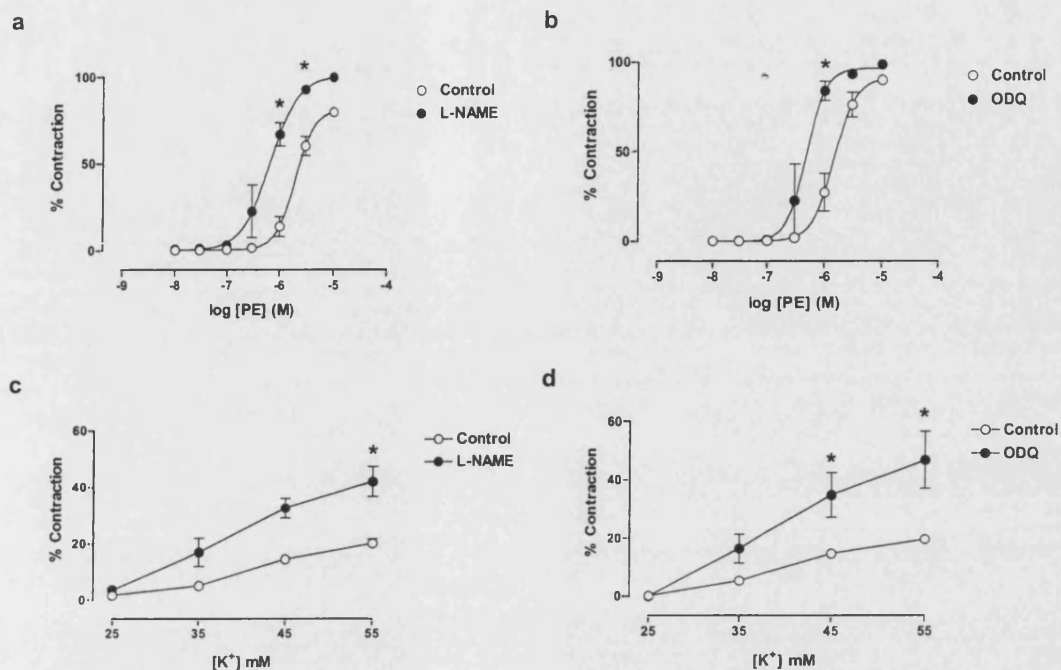


Figure 5.2 Effect of L-NAME and ODQ on PE- and K⁺-evoked contraction.

Phenylephrine concentration-response curves were shifted to the left in the presence of L-NAME (100 μ M, a, pD_2 values for control and L-NAME treated arteries were 5.7 ± 0.0 and 6.2 ± 0.1 respectively, $P < 0.05$, $n = 3$) and ODQ (10 μ M, b, pD_2 values for control and ODQ treated arteries were 5.8 ± 0.1 and 6.3 ± 0.1 respectively, $P < 0.05$, $n = 4$). Both L-NAME and ODQ also significantly augmented contraction to K⁺ (c and d, $P < 0.05$, $n = 3$ & 3 respectively). Values are means \pm S.E.M from n arteries. Analysis by Students' paired t-test to compare pD_2 values, and one-way ANOVA with Bonferroni's post-test to compare individual points between curves. Significant differences between points indicated by *, $P < 0.05$.

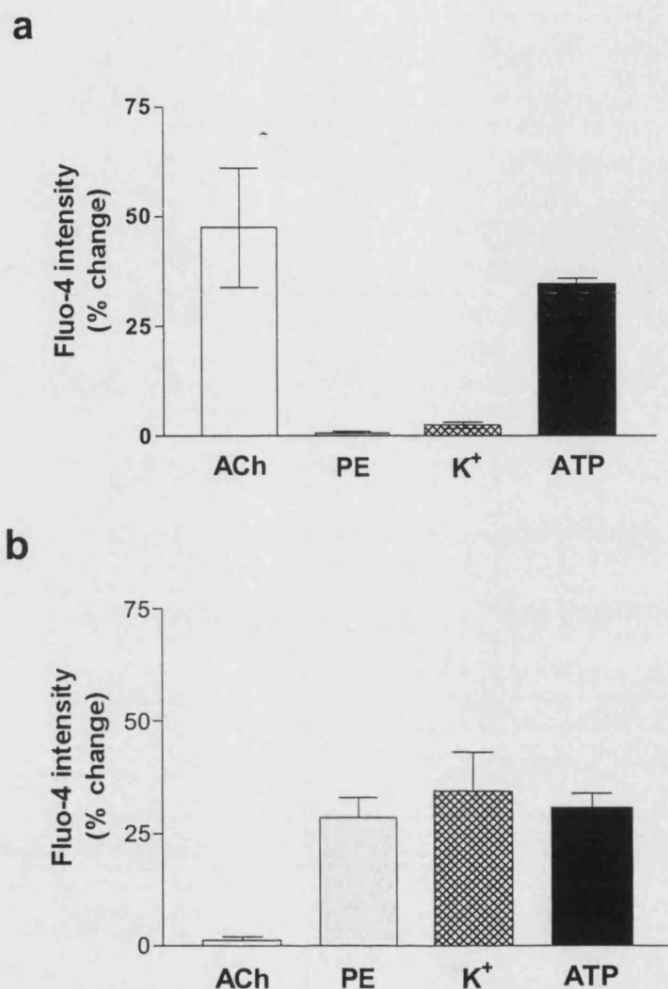


Figure 5.3 Responses to agonist stimulation by freshly isolated rat mesenteric endothelial and smooth muscle cells loaded with fluo-4.

Endothelial cells (a, $n = 3$, 14 cells) loaded with fluo-4 (50 μM) responded with an increase in fluorescence intensity to stimulation with ACh (10 μM) and ATP (10 μM), while PE (10 μM) and K⁺ (40 mM) were without effect. Smooth muscle cells (b, $n = 3$, 3 cells) responded with an increase in fluorescence intensity to stimulation with PE, K⁺ and ATP, while ACh was without effect. Values are mean \pm S.E.M from n animals.

5.3.3 Effect of prostanoid synthesis inhibition, BK_{ca} inhibition and gap junction uncoupling agents on L-NAME-evoked contractions of phenylephrine pre-stimulated arteries.

The presence of indomethacin (2.8 μ M), iberiotoxin (IbTx, 100 nM) and the gap junction uncouplers carbenoxolone (CBX, 100 μ M) and Gap 27 (300 μ M) were without effect on the magnitude of the L-NAME contraction following stimulation with PE ($P > 0.05$ for all, $n = 3, 3, 6$ and 3 respectively, Table 5.2).

5.3.4 Phenylephrine- and acetylcholine-evoked ATP release from rat mesenteric arteries.

Stimulation with PE (10 μ M) and ACh (10 μ M) evoked the release of ATP from intact mesenteric arteries (427 ± 172 pmol/l, $n = 4$ and 507 ± 159 pmol/l, $n = 4$, respectively, Figure 5.4). PE stimulated release was abolished by the presence of glibenclamide (10 μ M, 39 ± 15 pmol/l, $P < 0.05$, $n = 4$) and unaffected by the vesicle fusion inhibitor N-ethylmaleimide (NEM, 1 μ M, 570 ± 267 pmol/l, $P > 0.05$, $n = 3$, Figure 5.4a). In contrast, ACh evoked release was abolished by NEM (64 ± 19 pmol/l, $P < 0.05$, $n = 4$) and unaffected by glibenclamide (444 ± 220 pmol/l, $P > 0.05$, $n = 3$, Figure 5.4b). The presence of ATP was not detected in samples of MOPS buffer following the addition of PE or ACh in the absence of tissue or following addition of an equivalent volume of MOPS buffer to that added for PE and ACh in the presence of tissue. The presence of

DMSO (glibenclamide solvent), was without effect on ATP release evoked by PE (493 ± 60 pmol/l, $P > 0.05$, $n = 3$).

Table 5.2 Contractions evoked by L-NAME in rat mesenteric arteries pre-contracted with PE.

	PE
<i>Control</i>	53.8 ± 4.7 (<i>n</i> = 8)
<i>Carbenoxolone</i>	47.2 ± 6.7 (<i>n</i> = 6)
<i>Gap 27</i>	36.3 ± 6.1 (<i>n</i> = 3)
<i>Iberiotoxin</i>	45.3 ± 0.9 (<i>n</i> = 3)
<i>Indomethacin</i>	51.9 ± 3.8 (<i>n</i> = 3)
<i>Glibenclamide</i>	15.0 ± 2.3 (<i>n</i> = 3)*

Arteries were initially contracted to $\leq 25\%$ of maximum contraction with phenylephrine (PE, 300 nM – 3 μ M) in absence (control) or presence of the above agents and were allowed to plateau before the addition of L-NAME (100 μ M). Carbenoxolone (100 μ M), Gap 27 (300 μ M), iberiotoxin (100 nM) and indomethacin (2.8 μ M) were without effect on L-NAME evoked contraction, while glibenclamide (10 μ M) significantly attenuated the response. Values are expressed as percentage of maximum contraction and are means \pm S.E.M from *n* arteries. Analysis by one-way ANOVA with Bonferroni's post test. Significant differences against L-NAME control indicated by * ($P < 0.05$).

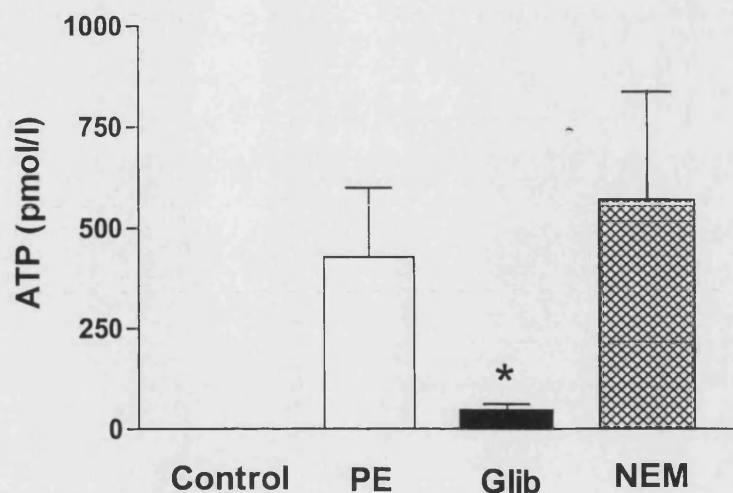
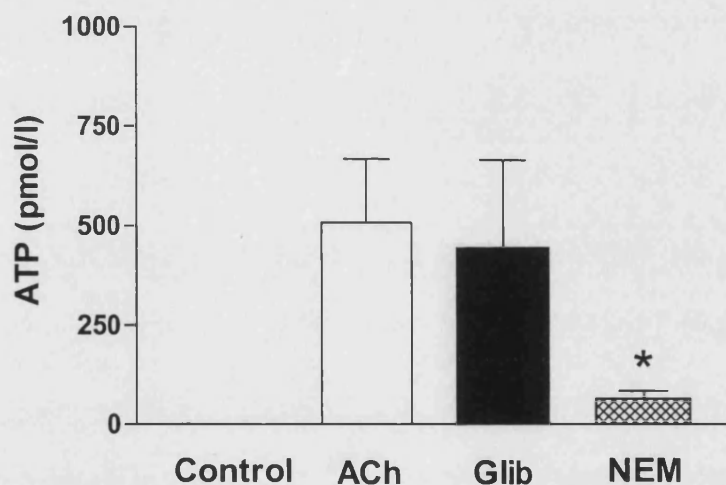
a**b**

Figure 5.4 PE and ACh stimulated ATP release from rat mesenteric arteries.

Stimulation with phenylephrine (PE, a, 10 μ M) and acetylcholine (ACh, b, 10 μ M) evoked release of ATP from rat mesenteric arteries. PE evoked release was abolished by glibenclamide (10 μ M, a, $P < 0.05$, $n = 4$) and unaffected by NEM (1 μ M, a, $P > 0.05$, $n = 3$). In contrast, NEM abolished ACh evoked ATP release (b, $P < 0.05$, $n = 4$) and glibenclamide was without effect (b, $P > 0.05$, $n = 3$). In the absence of PE ($n = 4$) or ACh ($n = 4$) no ATP could be measured. Values are means \pm S.E.M from n separate experiments. Analysis by one-way ANOVA with Bonferroni's post-test.

5.3.5 Effect of glibenclamide on L-NAME-evoked augmentation of phenylephrine contraction and relaxation to DEA NONOate.

The presence of glibenclamide (10 μ M) inhibited the L-NAME (100 μ M) induced leftward shift of the PE concentration-response curve seen under control conditions (Figures 5.2a and 5.5) and significantly attenuated L-NAME evoked contraction following pre-contraction with PE ($P < 0.05$, $n = 3$, Table 5.2). Glibenclamide was without effect on relaxation to the NO donor DEA NONOate (pD_2 values for control and DEA NONOate treated arteries were 7.3 ± 0.0 and 7.4 ± 0.0 respectively, $P > 0.05$, $n = 4$, Figure 5.6).

5.3.6 Effect of P_{2x1} receptor desensitisation on phenylephrine-evoked contraction.

P_{2x1} receptor desensitisation with α,β -methylene-ATP (α,β -MeATP, 10 μ M) induced a rightward shift of the PE concentration-response curve (pD_2 values for control and α,β -MeATP treated arteries were 5.7 ± 0.0 and 5.4 ± 0.1 respectively, $P < 0.05$, $n = 4$, Figure 5.7).

5.3.7 ATP stores in mesenteric smooth muscle and endothelial cells

Compartmentalized cytosolic fluorescence observed in isolated smooth muscle ($n = 3$) and endothelial cells ($n = 2$) incubated with quinacrine (100 nM) revealed the presence of vesicular ATP stores in both cell types (Figure 5.8). Simultaneous labelling with the mitochondrial marker mitofluor (100 nM) was not co-localized with that of quinacrine.

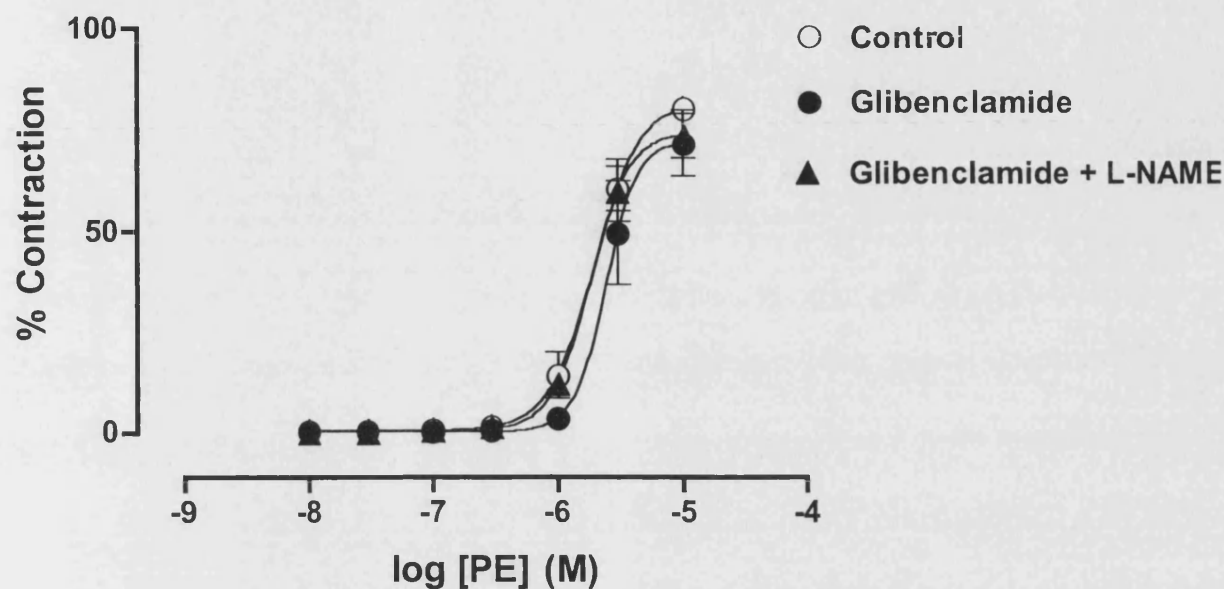


Figure 5.5 Effect of glibenclamide on L-NAME-evoked augmentation of PE contraction.

In the presence of glibenclamide (10 μ M), L-NAME (100 μ M) failed to evoke a leftward shift in the phenylephrine concentration-response curve (pD_2 values for control and glibenclamide plus L-NAME treated arteries were 5.7 ± 0.0 and 5.7 ± 0.0 respectively, $P > 0.05$, $n = 3$). Glibenclamide itself was without effect on phenylephrine-evoked contraction (pD_2 5.6 ± 0.1 , $P > 0.05$, $n = 3$). Values are means \pm S.E.M from n arteries. Analysis by Students' paired t-test to compare pD_2 values, and one-way ANOVA with Bonferroni's post-test to compare individual points between curves. Significant differences between points indicated by *, $P < 0.05$.

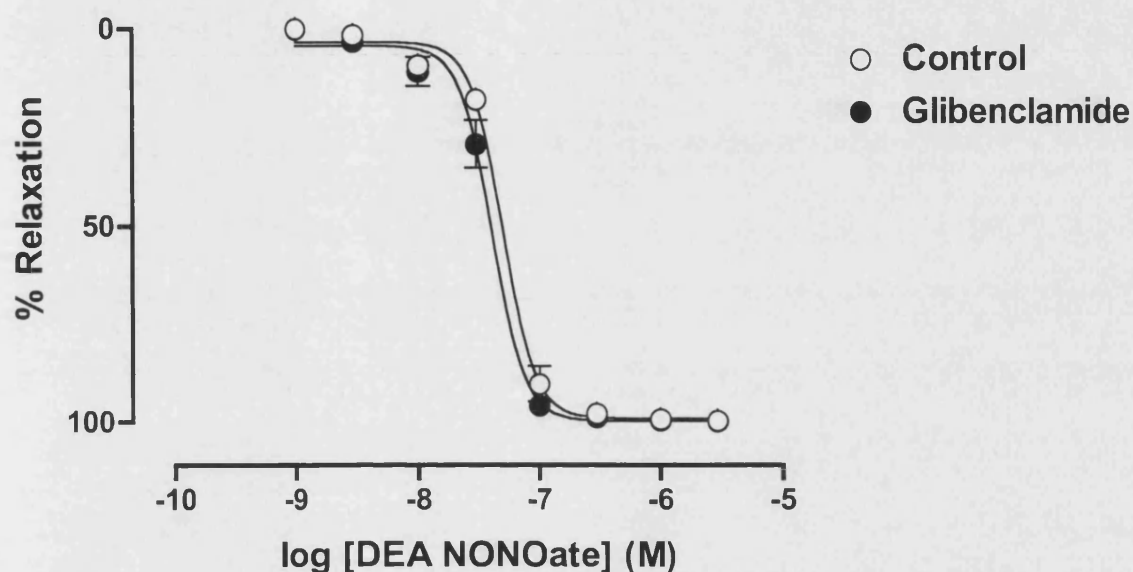


Figure 5.6 Effect of glibenclamide on relaxation evoked by DEA NONOate.

Cumulative concentration-response curve to DEA NONOate (1 nM – 10 μ M) in rat mesenteric arteries contracted with phenylephrine. Relaxation to DEA NONOate was unaffected by the presence of glibenclamide (10 μ M, pD_2 values for control and glibenclamide treated arteries were 7.3 ± 0.0 and 7.4 ± 0.0 respectively, $P > 0.05$, $n = 4$). Values are mean \pm S.E.M from n arteries. Analysis by Students' paired t-test to compare pD_2 values, and one-way ANOVA with Bonferroni's post-test to compare individual points between curves. Significant differences between points indicated by *, $P < 0.05$.

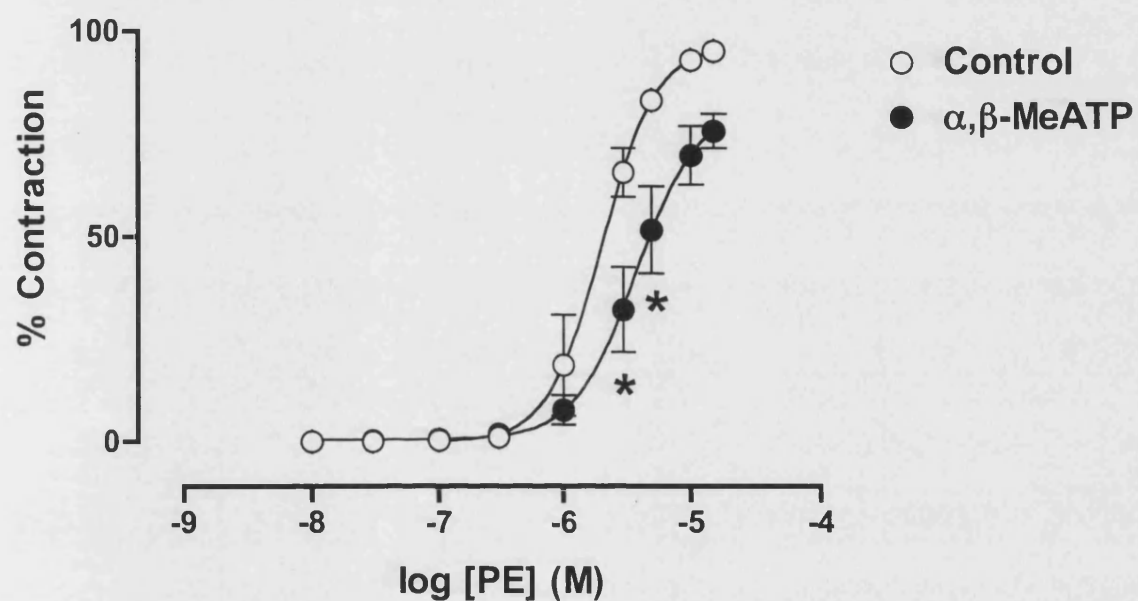


Figure 5.7 Effect of α,β -methylene-ATP on contraction evoked by PE.

The phenylephrine concentration-response curve was shifted to the right in the presence of α,β -methylene-ATP (α,β -MeATP, 10 μ M, pD_2 values for control and α,β -MeATP treated arteries were 5.7 ± 0.0 and 5.4 ± 0.1 respectively, $P < 0.05$, $n = 4$). Values are mean \pm S.E.M from n arteries. Analysis by Students' paired t-test to compare pD_2 values, and one-way ANOVA with Bonferroni's post-test to compare individual points between curves. Significant differences between points indicated by *, $P < 0.05$.

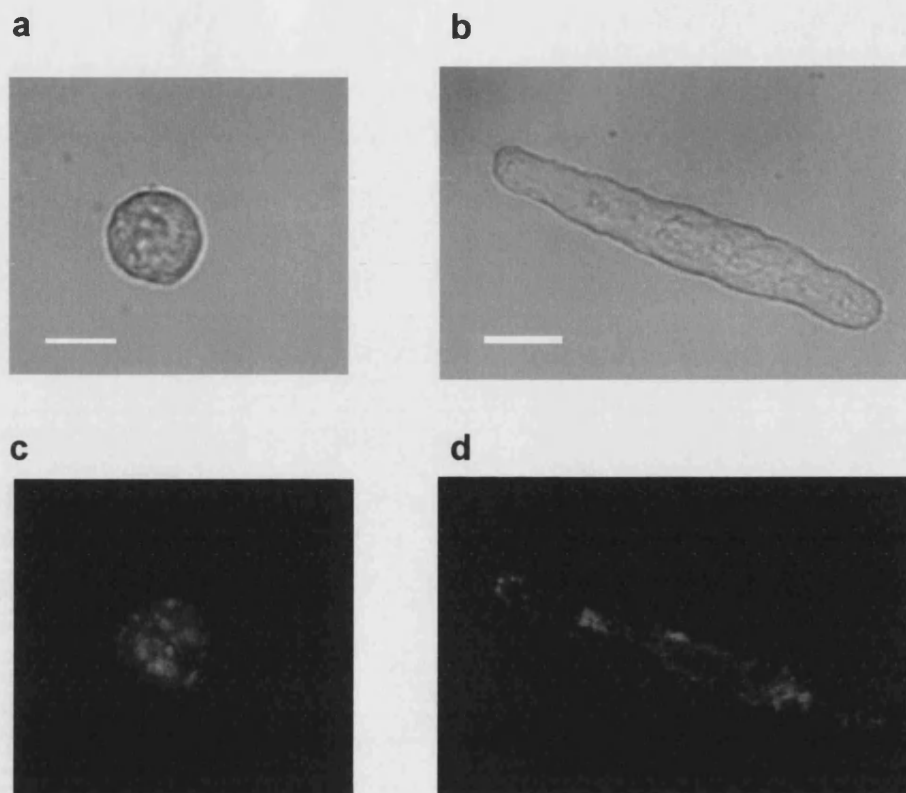


Figure 5.8 Quinacrine and mitofluor labelling of freshly isolated rat mesenteric smooth muscle and endothelial cells.

Representative images. Bright field images of freshly isolated endothelial (a) and smooth muscle (b) cells. c and d) Fluorescence images of cells a and b respectively following incubation with quinacrine (100 nM, barrier filter BA505-525) and mitofluor (100 nM, barrier filter BA610F). The granular quinacrine staining (green) observed in both cell types was not co-localized with that of the mitochondrial marker mitofluor (red). Bars = 20 μ m.

5.4 Discussion

The ability of L-NAME to augment contraction to PE in rat mesenteric arteries is consistent with the findings of Dora *et al.*, 2000 and suggests that α_1 -adrenoceptor stimulation is associated with the generation of NO. The ability of the soluble guanylate cyclase inhibitor ODQ to also augment PE induced contraction, is further confirmation that the leftward shift of the PE concentration-response curve is resulting from the inhibition of NO mediated relaxation and that this relaxation is cGMP dependent. Non-receptor dependent contraction induced by K^+ was similarly augmented by L-NAME and ODQ and suggested that elevations in smooth muscle cell calcium may be a general mechanism for endothelial NO production. In PE pre-contracted arteries, removal of the endothelium abolished L-NAME evoked contraction and suggested that the release of NO in response to this agonist was entirely endothelial in origin. The inability of endothelial removal to affect L-NAME evoked contraction in K^+ pre-contracted arteries, suggested that the endothelium was not the source of NO released in response to K^+ stimulation. Further contraction of K^+ but not PE stimulated arteries by the nNOS inhibitor PLA (Cooper *et al.*, 2000; Zhang *et al.*, 1997), confirmed that K^+ was able to stimulate the release of neuronal NO. Contraction to L-NAME following incubation with PLA in K^+ pre-contracted arteries, suggested additional release of endothelial NO by K^+ , or that the concentration of PLA used was insufficient to completely block nNOS. The endothelium-independent release of NO by K^+ limited the usefulness of this agonist in the study of smooth muscle evoked endothelial NO release.

Elevations in endothelial cell Ca^{2+} stimulate the production and release of NO (Fleming & Busse, 1999). While there is no evidence for the presence of calcium mobilizing α_1 -

adrenoceptors on vascular endothelium, this has been a proposed explanation for α_1 stimulated NO release (Boer *et al.*, 1999; Kaneko & Sunano, 1993; Tuttle & Falcone, 2001). The possibility that in this study, PE was directly stimulating mesenteric endothelial cells to evoke a rise in $[Ca^{2+}]_i$ was dismissed, since PE was unable to evoke an increase in fluo-4 fluorescence in isolated endothelial cells loaded with the Ca^{2+} sensitive dye. The ability of PE to directly stimulate mesenteric smooth muscle cells was confirmed by an evoked response in a similar set of experiments.

Under isometric conditions, the amplitude of the L-NAME induced contraction has been shown to increase with increasing levels of pre-contraction in rabbit thoracic aorta (Fleming *et al.*, 1999). This led the authors to propose that isometric contraction *per se* initiates a signalling pathway leading to endothelial NO production, although an alternative mechanism involving unidentified intercellular signalling molecules between endothelial cells and the underlying smooth muscle was not excluded. In contrast to the findings of Fleming *et al.* (1999), the level of pre-contraction with PE did not correlate with the amplitude of the subsequent contraction evoked by L-NAME in this study. Combined with the finding that L-NAME also evoked contraction in PE stimulated arteries under isobaric conditions, isometric contraction *per se* is not the only stimulus for contraction-evoked endothelial NO production in rat mesenteric arteries.

The necessity for an increase in endothelial cell $[Ca^{2+}]_i$ to evoke vasoconstriction induced NO release has been demonstrated by the ability of NA and PE to elevate endothelial cell Ca^{2+} and potentiate constriction of cremaster arteries after endothelial denudation or NOS inhibition (Tuttle & Falcone, 2001). The inability of endothelial denudation or NOS inhibition to potentiate $PGF_{2\alpha}$ constriction was concluded to be a

likely consequence of the failure of $\text{PGF}_{2\alpha}$ to elevate endothelial cell Ca^{2+} despite elevating smooth muscle cell Ca^{2+} (Tuttle & Falcone, 2001). This would suggest that an increase in smooth muscle Ca^{2+} *per se* is not the stimulus for increasing endothelial cell Ca^{2+} and that vasoconstriction evoked NO release may be an α_1 -adrenoceptor dependent mechanism. The finding in hamster cheek arterioles that both PE and KCl stimulate an expected rise in smooth muscle cell Ca^{2+} accompanied by an elevation in endothelial cell Ca^{2+} and NO synthesis, has led others to conclude that elevation of smooth muscle cell Ca^{2+} is the stimulus for increasing endothelial cell Ca^{2+} and the generation of NO (Dora *et al.*, 1997). Myoendothelial gap junctions may provide a signalling pathway for the communication of changes in the $[\text{Ca}^{2+}]_i$ between smooth muscle and endothelial cells. Potentially this could involve the passive diffusion of Ca^{2+} or IP_3 from the smooth muscle to the endothelium along their concentration gradients. Indeed, evidence exists to support the gap junctional transfer of these second messengers between cells (Saez *et al.*, 1989; Sanderson *et al.*, 1990; Yule *et al.*, 1996) and the existence of myoendothelial gap junctions in rat mesenteric arteries (Sandow *et al.*, 2002). In support of a role for these channels in the communication of changes in $[\text{Ca}^{2+}]_i$ between smooth muscle and endothelial cells, PE evoked increases in endothelial cell Ca^{2+} have been shown to be attenuated by the gap junction uncoupler palmitoleic acid (Schuster *et al.*, 2001). In the present study, the gap junction uncoupling agents carbenoxolone and Gap 27 were without effect on the magnitude of the of the L-NAME evoked contractions in PE pre-contracted arteries, suggesting that these channels are not involved in PE evoked NO release in rat mesenteric arteries.

Ruling out the involvement of a direct signalling pathway involving myoendothelial gap junctions, other potential mechanisms by which eNOS may be activated in response to

smooth muscle stimulation with PE, include the extracellular diffusion of an unidentified signalling factor from the smooth muscle to the endothelium. Potential candidates for this role include the prostanoids, which have been implicated in the attenuation of angiotensin II evoked contraction in vivo (Schuijt *et al.*, 2001), K^+ which has been suggested to efflux from vascular smooth muscle *via* BK_{ca} in response to elevations in $[Ca^{2+}]_i$ (Dora *et al.*, 2000; Dora *et al.*, 2002; Richards *et al.*, 2001) and can act to hyperpolarize endothelial cells (Crane *et al.*, 2003; Doughty *et al.*, 2001) potentially resulting in an increasing electrical gradient for Ca^{2+} influx (Cannell *et al.*, 1989) and ATP, which can be released from smooth muscle cells by calcium mobilizing agonists (Katsuragi *et al.*, 2002) and can stimulate the production of NO from vascular endothelial cells (Stanford *et al.*, 2001). The inability of the cyclo-oxygenase inhibitor indomethacin and the BK_{ca} blocker iberiotoxin to have an effect on the magnitude of L-NAME evoked contraction, argues strongly against the involvement of prostanoids or K^+ in eNOS activation.

The finding that ATP was released from rat mesenteric arteries in response to PE stimulation, is the first reported demonstration of a Ca^{2+} -mobilising agonist evoking ATP release from vascular smooth muscle. Within renal epithelium, ATP release across the apical membrane in response to Ca^{2+} -mobilising agonist stimulation has been attributed to exocytosis of ATP storage vesicles (Schwiebert, 2001). Due to its high affinity for ATP, quinacrine has been used in a number of studies to locate possible intracellular stores of this purine and has revealed the presence of vesicular ATP stores in human umbilical vein endothelial cells, ocular ciliary epithelial cells and rat pancreatic acini (Bodin & Burnstock, 2001; Mitchell *et al.*, 1998; Sorensen & Novak, 2001). In the present study, the compartmentalized cytosolic fluorescence observed in

isolated smooth muscle cells incubated with this compound, revealed the presence of vesicular ATP stores in mesenteric smooth muscle. The absence of co-localized staining between quinacrine and the mitochondrial marker mitofluor, indicated the ATP containing compartments to be distinct from mitochondria. However, the lack of effect of the membrane vesicle fusion inhibitor NEM (Sorensen *et al.*, 2002; Sorensen, 2004) upon PE evoked ATP release, suggested that exocytosis was not the mechanism for agonist-stimulated smooth muscle ATP release.

Evidence for ABC protein-mediated ATP release exists in numerous cell types in response to various stimuli (Ballerini *et al.*, 2002; Hassessian *et al.*, 1993; Payen *et al.*, 2001; Roman *et al.*, 2001; Roman *et al.*, 1997; Sprague *et al.*, 1998). ABC proteins are believed to either transport ATP themselves, or stimulate the activity of separate ATP channels or pumps (Demolombe & Escande, 1996; Guidotti, 1996; Schwiebert, 1999). In addition to its common use as an inhibitor of the sulphonylurea receptor, glibenclamide has been shown to be a potent inhibitor of other members of the ABC protein family (Golstein *et al.*, 1999; Sheppard & Robinson, 1997) and has been used as a general inhibitor of ABC proteins (Knight *et al.*, 2002; Payen *et al.*, 2001). In rat mesenteric arteries, abolition of PE evoked ATP release in the presence of glibenclamide, implicated ABC proteins in the trans-membrane transport of this purine from the smooth muscle. Taken together with the finding that glibenclamide significantly attenuated L-NAME evoked contraction in PE pre-stimulated arteries, these data suggest that PE induced ATP release from smooth muscle cells *via* ABC proteins mediates NO production and vasodilatation, possibly by activating endothelial P₂ receptors (Ralevic & Burnstock, 1998). Indeed, consistent with the idea that glibenclamide inhibits PE induced NO release, incubation with the sulphonylurea

blocked the leftward shift of the PE concentration-response curve induced by L-NAME under control conditions. The finding that glibenclamide was without effect on relaxation mediated by NO donor DEA NONOate, suggested that the inhibitory effect of glibenclamide on L-NAME evoked augmentation of contraction was not owing to blockade of the mechanism for NO-mediated relaxation rather than PE-evoked NO release. Glibenclamide itself did not induce a leftward shift of the PE concentration-response curve, which would be expected if PE-evoked NO release had been blocked. This suggested the possibility that glibenclamide may also be attenuating smooth muscle contraction and thereby masking the expected leftward shift of the curve. Due to the presence of P_2 receptors on vascular smooth muscle (Ralevic & Burnstock, 1998), PE induced ATP release from smooth muscle cells would be expected to induce vasoconstriction in addition to vasodilatation resulting from activation of P_2 receptors on the endothelium. In vascular smooth muscle the predominant receptor subtype for ATP is a rapidly desensitizing ligand-gated ion channel P_{2x1} (Hansen *et al.*, 1999; North & Surprenant, 2000). Desensitization of the P_{2x1} receptor with α,β -MeATP induced a rightward shift of the PE concentration-response curve indicating that PE stimulation of mesenteric smooth muscle also results in activation of P_{2x1} receptors to augment α_1 -adrenoceptor mediated contraction. Simultaneous activation of P_2 receptors with opposing vasodilator and vasoconstrictor responses may explain why glibenclamide failed to induce the expected leftward shift of the PE concentration-response curve. The presence of compartmentalized ATP stores within mesenteric smooth muscle cells, yet an apparent absence of an exocytotic release mechanism for PE evoked trans-membrane ATP transport, suggests that the ATP stores represent a release pathway for other types of stimuli or serve another unknown function.

According to this model, PE evoked ATP release from smooth muscle cells activates endothelial P₂ receptors to induce an increase in [Ca²⁺]_i and release of NO. If indeed an increase in [Ca²⁺]_i is the stimulus for ATP release, being itself a Ca²⁺-mobilising agonist, the ATP stimulated rise in endothelial [Ca²⁺]_i may induce further release of ATP from the endothelium. Indeed, Ca²⁺-mobilising agonists including ATP have been demonstrated to release ATP from vascular endothelial cells (Bodin & Burnstock, 1996; Schwiebert *et al.*, 2002). ATP release from rat mesenteric arteries in response to stimulation with the endothelial selective Ca²⁺-mobilising agonist ACh, suggested that an ATP evoked rise in endothelial cell [Ca²⁺]_i may indeed stimulate ATP release from these cells. In contrast to PE evoked ATP release from smooth muscle, ACh evoked ATP release occurred by exocytosis and was not dependent upon a mechanism involving ABC proteins. Quinacrine labelling confirmed the presence of compartmentalized ATP stores distinct from mitochondria within isolated endothelial cells. The possibility of ATP stimulated ATP release also exists for mesenteric smooth muscle cells and has been demonstrated in smooth muscle of guinea pig ileum and vas deferens (Katsuragi *et al.*, 1991).

5.5 Conclusions

In summary, α_1 -adrenoceptor stimulation of rat mesenteric arteries may be associated with the release of ATP from the smooth muscle by a mechanism involving ABC proteins. By activating endothelial P_2 receptors, ATP released by the smooth muscle stimulates the production of NO and smooth muscle relaxation. ATP evoked ATP release within the endothelium may perpetuate this response. In this way, ATP and/or its metabolites behave as smooth muscle derived relaxing factor(s) in this artery. The physiological role of this negative feedback mechanism is possibly to maintain adequate tissue perfusion in the presence of a strong contractile stimulus and may be a general regulatory mechanism for vascular contraction (Figure 5.9).

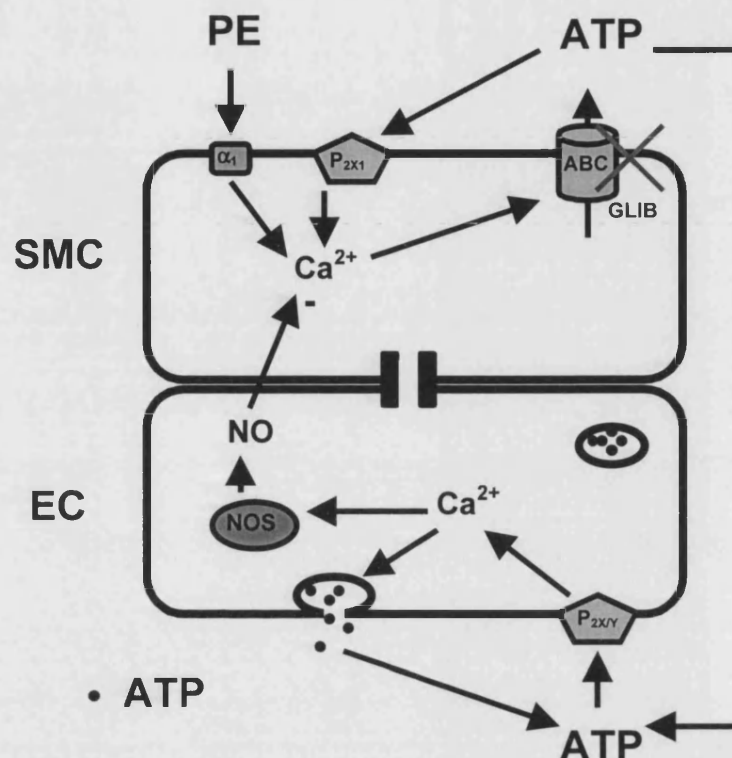


Figure 5.9 Schematic diagram of the proposed mechanism for PE-evoked NO release in rat mesenteric arteries.

α_1 -receptor stimulation of rat mesenteric smooth muscle evokes an increase in smooth muscle $[Ca^{2+}]_i$ and efflux of ATP from the smooth muscle by a mechanism involving ABC proteins. The released ATP and/or its metabolites can activate smooth muscle and endothelial P_2 receptors to evoke a further rise in smooth muscle $[Ca^{2+}]_i$ and stimulate a rise in endothelial $[Ca^{2+}]_i$, resulting in the production and release of NO. Increases in endothelial cell Ca^{2+} may also stimulate release of ATP to further activate endothelial and smooth muscle P_2 receptors.

General summary and future directions

General summary and future directions

The findings presented in this thesis demonstrate the requirement for gap junctions in the communication of changes in $[Ca^{2+}]_i$ between rat aortic endothelial cells, while in contrast, suggest an extracellular signalling mechanism to underlie vasoconstriction-evoked NO release in rat mesenteric arteries. In certain circumstances, transmission of electrical signals between rat mesenteric smooth muscle and endothelial cells involved both gap junctional and extracellular signalling mechanisms operating in parallel. Under these conditions, although still functional, gap junctions were not essential for endothelium-dependent hyperpolarization of mesenteric smooth muscle.

Application of a mechanical stimulus to a single rat aortic endothelial cell in a confluent monolayer, evoked an increase in $[Ca^{2+}]_i$ within the stimulated cell which spread to neighbouring non-stimulated cells in a gap junction-dependent manner. The finding that the spreading Ca^{2+} response was not dependent upon a rise in $[Ca^{2+}]_i$ within the stimulated cell and could be abolished by PLC inhibition and intracellular Ca^{2+} store depletion, suggested a Ca^{2+} -mobilizing messenger, other than Ca^{2+} itself to underlie the communication of changes in $[Ca^{2+}]_i$ between these cells. Future directions for research arising from these observations include:

i) Further investigation of the nature of the intercellular messenger. Experiments examining the effect of IP_3 R antagonists on the intercellular spread of changes in $[Ca^{2+}]_i$ between these cells may provide supporting evidence for the involvement of IP_3 in this response.

ii) Extending this work to rat mesenteric artery endothelial cells to investigate whether changes in $[Ca^{2+}]_i$ are also communicated between endothelial cells of resistance arteries. This work could be carried out on freshly isolated 'sheets' of rat mesenteric endothelial cells for which an isolation procedure has been established in this laboratory.

The finding that gap junctions were the only apparent mechanism for EDHF-mediated relaxation of rat mesenteric arteries at high levels of contraction, while at low levels they were not, suggested that the intensity of smooth muscle contraction influences the mechanism by which EDHF-type responses occur, with the dependence on gap junctional signalling increasing as the ability of extracellular K^+ to evoke smooth muscle relaxation decreases. This does not preclude the importance of gap junctions at all levels of contraction. These observations suggest the following possibilities for further investigation:

- i) Identifying the mechanisms by which extracellular K^+ evokes smooth muscle relaxation of rat mesenteric arteries and whether pharmacological blockade of relaxation to extracellular K^+ will result in gap junction-dependent EDHF-mediated relaxation.
- ii) The use of a combination of connexin-mimetic peptides to fully block EDHF-mediated relaxation and support the findings with carbenoxolone.

Activation of smooth muscle α_1 -adrenoceptors with phenylephrine stimulated the release of endothelial nitric oxide in rat mesenteric arteries. The finding that inhibition of phenylephrine-evoked ATP release by glibenclamide from mesenteric smooth muscle blocked L-NAME-induced augmentation of phenylephrine contraction, suggested extracellular ATP signalling, independent of gap junctions, underlies phenylephrine-evoked NO release in these arteries. Further study based on these observations could include:

- i) Establishing whether the signalling molecule is ATP or a metabolite.
- ii) Identifying the endothelial purinergic receptors stimulated by the above.
- iii) Repetition of experiments with other contractile agonists to determine whether vasoconstriction-evoked NO release from mesenteric endothelial cells is a general phenomenon or solely α_1 -adrenoceptor dependent.
- iv) Identifying which ABC proteins are present in mesenteric smooth muscle cells and the precise nature of the stimulus for ABC protein-mediated ATP release.
- v) Investigating the role of the observed ATP stores in mesenteric smooth muscle and endothelial cells.

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Appendices

Appendix 1

Krebs-Henseleit buffer

Compound	Concentration (mM)
NaCl	119.0
KCl	4.7
CaCl ₂	2.5
KH ₂ PO ₄	1.18
MgSO ₄	1.2
NaHCO ₃	25.0
Glucose	11.0

pH adjusted to 7.4 by bubbling with 95% O₂/ 5% CO₂.

Appendix 2

MOPS buffer

Compound	Concentration (mM)
NaCl	145.0
KCl	4.7
CaCl ₂	2.0
MgSO ₄	1.17
MOPS	2.0
NaH ₂ PO ₄	1.2
Glucose	5.0
Pyruvate	2.0
EDTA	0.02
NaOH	2.75

pH adjusted to 7.4 with NaOH.

Appendix 3

Low Ca²⁺ PSS

Compound	Concentration (mM)
NaCl	136.0
KCl	5.6
MgCl ₂	0.47
NaHCO ₃	4.17
NaH ₂ PO ₄	0.44
Na ₂ HPO ₄	0.42
HEPES	10.0
CaCl ₂	0.10

pH adjusted to 7.4 with NaOH.

Appendix 4

Ca²⁺ free PSS

Compound	Concentration (mM)
NaCl	124.7
KCl	4.8
MgCl ₂	3.7
KH ₂ PO ₄	1.2
HEPES	10.0
EGTA	1.0
Glucose	11.0

pH adjusted to 7.4 with NaOH.